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ERRATA.

P. 146, l. 3, "0.1" should read "0.01."

P. 160, Chart 2, l. 4, "0.033 mg." should read "0.016."

P. 274, l. 17, "after incubating, 18-24 colonies" should read "after incubating 18-24 hours, colonies."

P. 276, l. 14, "inoculation" should read "influence."

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No. 1

AVIAN TUBERCULOSIS.*†

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(From the College of Agriculture, University of Wisconsin, Madison, Wisconsin.)

Avian tuberculosis has not attracted especial attention in this country until quite recently. It was reported by Pernot¹ in 1900, but previously a disease had been described to which the term "tuberculosis" had been applied, although, as far as we are aware, no demonstration of the bacilli in lesions was made before the work of Pernot. Within recent years it has attracted greater attention, and is coming to be recognized as an economic problem in the poultry industry. It seems probable that the disease has spread rapidly within the last few years, due to the great increase in the sale of birds for breeding purposes and in the sale of eggs for hatching, and that this increase in avian tuberculosis has been the active factor in bringing the disease to the attention of experiment station workers. Tubercle bacilli were not found in any avian tissue submitted to this station for examination prior to 1906. During the last three years tubercular tissues have been received from many

* Received for publication May 5, 1913.

† Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

¹ Bull. 64, Oregon Exp. Sta.

sections of the state. From the information obtained in this and other ways it is apparent that avian tuberculosis is widespread in Wisconsin. A number of other stations have issued publications in which data concerning the distribution of the disease is given.¹ The number of inquiries answered in the poultry journals and in the agricultural papers, that without doubt refer to avian tuberculosis, is very considerable. All these facts indicate the widespread distribution of the disease in this country.

In Europe it was recognized before the discovery of the tubercle bacillus by Koch, and has long been a factor of importance, not only among farm poultry, but in the aviaries of the great zoölogical gardens. Koch and Rabinowitsch² have made an extensive study of the disease, using as their source of material the 459 birds that died in the Berlin garden from December, 1903, to August, 1905. Twenty-seven per cent were found to be tubercular. Of the 500 birds from the London garden, examined by Shattock, Seligmann, Dudgeon and Panthon³ 30 per cent were tubercular. The British Royal Commission on Tuberculosis has also studied in detail the characteristics of 14 cultures.⁴

The great losses occasioned by the disease in a limited time have been emphasized by Ward,⁵ Marshall,⁶ and Edwards.⁷ In two of the flocks studied by us, losses ranging from 33 to 50 per cent during less than one year were encountered. It seems probable, however, that such losses are exceptional, and that in most diseased flocks the losses are not sufficiently large to attract attention.

Characteristics of avian tuberculosis.—The birds showing visible symptoms of the disease are usually over one year old, but feeding experiments have shown that young chickens are easily infected. It is probable that under natural conditions it requires a number of months for the disease to make such progress in the individual bird as to become apparent. A rooster purchased by a poultry man from the station flock, which is free from tuberculosis, was placed in a flock subsequently found to be diseased. One year

¹ *Bull.* 161, California Exp. Sta., 1904; *Bull.* 193, Ontario Agri. Coll.; *Circular* 12, Michigan Exp. Sta., 1911.

² *Virch. Arch.*, 1907, Beihefte, 190, p. 246.

³ *Lancet*, 1907, 2, p. 1443.

⁴ *Final Report*, Part II, Appendix, 1911, 4, p. 167.

⁵ *Bull.* 161, Cal. Exp. Sta., 1904.

⁶ *Circular* 12, Mich. Exp. Sta., 1911.

⁷ *Bull.* 193, Ontario Agri. Coll., 1911.

later a postmortem examination showed that the bird was in the last stages of tuberculosis.

The first apparent symptom of the disease is the marked loss of flesh. The weight of some of the birds examined by us has been less than one-third that of the normal bird. The extreme to which the emaciation may go before death ensues is shown in Fig. 1, in which is presented a cross-section of the breast of a tubercular bird as compared with that of a healthy bird. Lameness is often



FIG. 1.—Extreme emaciation in avian tuberculosis. Cross sections of the breast of a healthy bird and a tubercular one.

noted. This condition may be due to diseased joints or to extensive changes in the viscera. The popular names, applied to the disease, such as “going light,” and “rheumatism” refer to these symptoms.

Avian tuberculosis is to be classed as a disease of the organs of the abdominal cavity rather than of the pleural cavity. This is shown in Table 1 in which is given the distribution of the lesions in the 29 fowls examined.

In Table 2, is presented a summary of the results obtained in our work as compared with those of Edwards,¹ Moore,² and Koch and Rabinowitsch.³

¹ Bull. 193, Ontario Agricultural College., 1911.

The Pathology of the Infectious Diseases of Animals, 1906, p. 189.

² *Op. cit.*

The figures with reference to lesions in the ovary are not accurate, except in our work and that of Edwards. No data was given by the other investigators as to the relative proportion of

TABLE 1.
DISTRIBUTION OF LESIONS IN VARIOUS ORGANS OF TUBERCULAR FOWLS.

The plus sign indicates the presence of lesions in a given organ.

Fowl	Died or killed	Liver	Spleen	Intes- tine	Kid- ney	Ovary	Mes- en- tery	Lungs	Pan- creas	Joints	Bones	Giz- zard
1	Killed.....	+	+	+
2	Killed.....	+	+
3	Killed.....	+	+
4	Killed.....	+	+	+
5	Killed.....	+	+	+
6	Killed.....	+	+	+
7	Died.....	+	+	+
8	Died.....	+	+	+
9	Killed.....	+	+	+
10	Died.....	+	+	+	+	..
11	Died.....	+	+	+	..	+
12	Killed.....	+	+	+
13	Killed.....	+	+	+
14	Killed.....	+	+	+
15	Died.....	+	+	..	+	+
16	Died.....	+	+	+
17	Died.....	+	+	+	+
18	Died.....	+	+
19	Died.....	+	+
20	Died.....	+	+	+
21	Died.....	+	+	+	+
22	Died.....	+	+	+	+
23	Killed.....	+	+	+	+	..
24	Killed.....	+	+	..	+	+
25	Killed.....	+	+	+
26	Killed.....	+	+
27	Died.....	+	+	+	+
28	Died.....	+	+	+	+
29	Killed.....	+	+	+	+	+	+	+	+

TABLE 2.
PERCENTAGE OF BIRDS SHOWING LESIONS IN VARIOUS ORGANS.

Organs Involved	Wisconsin	Edwards	Moore	Koch and Rabinowitsch
	Per cent	Per cent	Per cent	Per cent
Liver.....	97	99	76	85
Spleen.....	93	93	47	74
Intestine.....	60	61	35	44
Kidney.....	10	13	17	8
Ovary.....	14	13	6	4
Mesentery.....	17	21	23	..
Lungs.....	22	19	6	66
Bones.....	9	43	6	..

male and female birds examined. The work of Koch and Rabinowitsch was not confined to domestic fowls as in the case of the other investigators. The work of the German investigators has shown that probably all kinds of birds are susceptible to the disease.

The general appearance of the lesions has been described in many papers and texts. The tissue changes may be most extensive before death occurs. This was well shown in the case of Fowl 20, Table 1, in which the internal organs were badly diseased. The liver weighed 230 gms. and but little normal tissue remained in the spleen. The pronounced tissue changes, together with the enormous development of the bacilli in the lesions indicate the nontoxic nature of the disease.

The constant occurrence of tubercles on the walls of the intestine indicate that the organisms are eliminated in the feces and the

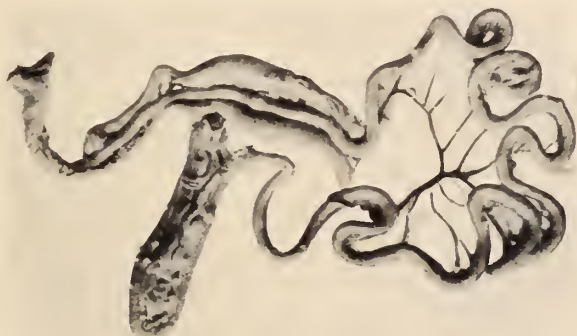


FIG. 2.—Tubercles on the intestines.

infection undoubtedly occurs by way of the alimentary tract. Weber and Bofinger¹ found it difficult to infect chickens by other methods than feeding. Intraperitoneal and intramuscular inoculation gave positive results only when large quantities of the culture were used. In our work intraperitoneal injections have always yielded positive results. A limited number of feeding experiments both with tubercular tissues and pure cultures have given positive results except in one case.

Rate of dissemination of avian tuberculosis.—An effort was made to obtain some evidence regarding the rate at which the disease may spread in a flock. For this purpose nine hens were obtained from a diseased flock. Four of the nine showed physical symptoms of tuberculosis. The above birds were placed with seven hens

¹ *Tuberculöse Arb. a.d. k. Gesundheitsamte*, 1904, 1, p. 83.

from a healthy flock. During the first month the birds were kept in a small room which was not cleaned during this period. One of the diseased birds died in 10 days. Postmortem examination showed tubercles on the intestines, and generalized tuberculosis of the abdominal cavity. Two others died in 26 and 46 days respectively. Both were extensively diseased and must have been eliminating tubercle bacilli. Of the remaining six hens from the tubercular flock which were killed one year later, five were found to be tubercular, but in none was the disease far advanced.

During the remainder of the year in which the healthy birds were associated with diseased ones, the flock was kept in a small colony house with a small yard. The house was not cleaned and the feed was scattered in the litter in order to make the exposure as extreme as possible. During the first two months of the experiment the exposure to infection was most complete, since the flock then contained birds in the last stages of tuberculosis. The first of the originally healthy hens was killed after eight months. The spleen and liver were tubercular and one nodule was found on one of the ceca. The remainder of the originally healthy hens were killed after having associated with the tubercular birds for about one year. Two of the six were tubercular, the others healthy. Thus three out of seven healthy hens acquired the disease. At the time they were killed the disease was not far advanced, and it is certain that the disease would not have progressed to a fatal termination in several months.

Transmission from flock to flock.—There would seem to be little doubt but that the diseased bird is the important agent in the spread of the disease from flock to flock. As shown in Table 1, the ovary is often involved. No examinations of eggs have been made by us. Mohler and Washburn examined a number of eggs laid by a tubercular hen and were able to demonstrate the presence of tubercle bacilli in one. More recently Higgins¹ reports on the bacilli in eggs from a diseased flock. Koch and Rabinowitch infected fertile eggs with avian tubercle bacilli. The eggs were incubated. One hatched; the chick died of tuberculosis in 75 days. From this chain of evidence there would seem to be little

¹ *Rept. Vet. Director General of Canada, 1912, p. 83.*

doubt but that the eggs from diseased birds may serve to introduce the disease into healthy flocks. The purchaser of cattle is able to protect his herd from infection by the intelligent use of the tuberculin test. The uselessness of the subcutaneous test for fowls has been shown by Ward and by Edwards. It is also claimed¹ that neither tuberculin from avian or mammalian bacilli will produce a reaction when dropped into the conjunctival sac or applied to the scarified skin. Dr. Van Es² of the North Dakota

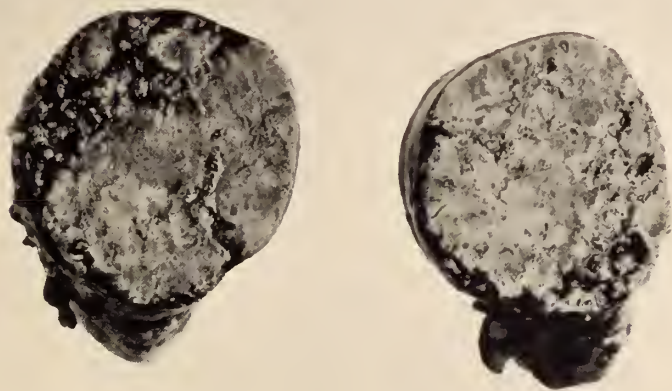


FIG. 3.—Tubercular spleen. The small amount of normal tissue is to be noted.

Experiment Station has made a number of tests of chickens by injecting avian tuberculin into the skin of the comb or wattles (intradermal test). Following his example two tests have been made by us on hens from a diseased flock. One reacted, the other did not. The results of the postmortem were in accord with the results of the test.

The avian tubercle bacillus.—Cultures directly from the tissues of hens have been isolated in nine of the 10 attempts made. Dorset's egg medium and glycerin agar have been used with success.

¹ Hutyra and Marek, *Pathology and Therapeutics of the Diseases of Domestic Animals*, translated by Mohler and Eichhorn, 1912, I, p. 614.

² Unpublished data.

The avian bacillus differentiates itself from the mammalian tubercle bacillus by the rapidity of growth on culture media, and the more pronounced color, yellow to pink, on solid media. In liquid media the growth is at first confined to the bottom, later a uniform turbidity is produced, and then the growth is again confined to the bottom, often forming a zoöglea-like mass, the broth remaining perfectly clear. If the depth of the fluid is not too great, the growth extends up the side of the flask until the surface is reached, when it spreads in a thin veil-like membrane which becomes thicker and wrinkled with age. The constancy with which surface membranes develop has been questioned by Koch and Rabinowitsch and by Weber and Bofinger. Our results are in agreement with those of O. Bang[†] who states that all of his cultures developed membranes, some appearing suddenly when the culture was old.

Meager growth has been obtained on media to which no glycerin was added.

A study of the effect of the avian tubercle bacillus on the reaction of glycerin broth has been made with a number of the cultures isolated as well as with one furnished us by Dr. S. F. Edwards of the Ontario Agricultural College. In a general way the avian tubercle bacillus is similar to the bovine type in that the reaction of the broth is changed from acid to alkaline. There has been almost no tendency for the reaction to again revert to an acid reaction. In Table 3 are given the results obtained with two

TABLE 3.
ALKALI PRODUCTION BY AVIAN TUBERCLE BACILLI.

Days Incubation	Culture 425.25	Check Flask	Days Incubation	Culture 425.15	Check Flask
	Per cent	Per cent		Per cent	Per cent
9	+1.55	+1.4	9	+1.5	+1.4
16	+1.0	+1.4	16	+1.0	+1.4
24	+1.2	+1.55	24	+0.95	+1.55
39	+1.0	+1.4	39	+0.55	+1.4
46	+1.0	+1.3	46	+0.55	+1.3
52	+0.75	+1.35	52	0.00	+1.35
59	+0.60	+1.35	59	0.00	+1.35
66	+0.80	+1.45	66	0.00	+1.45
73	+0.5	+1.4	173	0.00	+2.00
87	0.0	+1.8			
94	0.0	+1.8			

trials which are typical of all our results. Uninoculated flasks of the medium were incubated with the inoculated flasks. Dur-

[†] *Centraltbl. f. Bakteriöl.*, I, Orig., 1906, 43, p. 34.

ing the long period of incubation some desiccation took place; this accounts for the increase in acidity of the uninoculated flasks.

The determinations were made by diluting 5 c.c. of the culture, after heating the same in the steamer, with 45 c.c. of distilled water that had just been boiled, and titrating with N/20 NaOH, using phenolphthalein as an indicator. In no case has any marked degree of alkalinity been produced, the solution showing but a faint pink on addition of the indicator.

The avian tubercle bacillus stains more easily than does the human or bovine organism. A cold aqueous solution of methylene blue applied for a few moments imparts to the organisms sufficient color to render them easily visible. Preparations treated with 5 per cent nitric acid in 80 per cent alcohol after staining with carbol-fuchsin, and counter stained with methylene blue, show many cells with a blue color. Often the appearance may lead one to believe the culture to be impure, but subcultures on various types of media do not reveal any impurities.

In many ways the relation between certain of the acidfast, non-pathogenic bacilli and the avian tubercle bacillus seems to be much closer than between the avian and mammalian tubercle bacilli. This point will be discussed more in detail later.

Infection of mammals with avian tubercle bacilli.—The results obtained by previous investigators with guinea-pigs have been contradictory. It seems probable that some of the observations have been incorrect since death may be produced with no macroscopic lesions, but cultures, and microscopic examination may reveal a large number of tubercle bacilli in the various organs.

Moore¹ reports that guinea-pigs inoculated with avian tissues died in a very emaciated condition but showed no tubercular lesions. Edwards was unable to infect guinea-pigs with avian tissues. A portion of the guinea-pigs inoculated with pure cultures were killed in 100 days, and lesions were found. The remaining pigs were apparently healthy at the end of 10 months thus showing the disease to be of the non-progressive type. Straus and Gamaleia² were unable to produce anything but slight lesions. The same was

¹ *Jour. Med. Research*, 1904, 9, p. 521.

² *Arch. de méd. expér. et d'anat. path.* 1891, 3, p. 850.

true of Weber and Bofinger, and Maffucci¹ was unable to produce the disease on the second transfer through animals. On the other hand all but six of the 62 guinea-pigs inoculated by Koch and Rabinowitsch showed some effect, although in some that lived for months no lesions were visible on postmortem examination, but cultures gave positive results.

In our work 17 guinea-pigs were inoculated with avian tissues. Lesions were found in 15. Seven guinea-pigs were inoculated with pure cultures. Positive results were obtained in six. Four died in 27 to 63 days. Koch and Rabinowitsch noted that on passage through a series of animals the virulence diminished. The same observation has been made by us. Evidence indicative of this is presented in the following summary of the results of a series of inoculations:

Avian Tissue	{	Guinea-pig died in 28 days.	{	Guinea-pig died in 32 days.	{	Guinea-pig killed in 104 days. Tubercular.
		Guinea-pig killed in 46 days.		Guinea-pig died in 44 days.		Guinea-pig killed in 110 days. Tubercular.
				Guinea-pig died in 40 days.		

It is clear from the contradictory evidence that the virulence of the avian tubercle bacillus from different sources must vary widely.

In some cases the injection of rabbits with pure cultures produced death from generalized tuberculosis; in others the disease did not progress at all rapidly. In one rabbit, injected with avian tissues, the only lesions found on the 156th day were minute lesions on the cecum. In contradiction to most investigators except Koch and Rabinowitsch we have not found the avian organism more virulent for rabbits than for guinea-pigs.

Relation of avian tuberculosis to tuberculosis in swine.—It has been shown that the hog acquires tuberculosis most easily by way of the alimentary tract. We have found lesions in many of the internal organs six months after an animal had been given one feeding of milk infected with a pure culture of the bovine organism. The English Royal Commission found that avian tubercle bacilli would cause a non-progressive type of the disease in hogs. Mohler

¹ *Ztschr. f. Hyg. u. Infektionskrankh.*, 1892, 11, p. 445.

and Washburn¹ were able to infect hogs by feeding avian tissues. In our work four hogs have been fed on avian tubercular tissues. They were killed about six months after the first feeding. The lesions in all were confined to the cervical and mesenteric lymph glands. It is an open question whether the avian bacillus can produce such an extensive form of the disease in hogs as to cause condemnation of the carcass, but it is certain that it might cause such parts as the head to be rejected. Thus the disease becomes of economic importance other than in the poultry industry. Two hogs have been fed with pure cultures of the avian bacillus. That infection resulted from the inoculation has been shown by the tuberculin test with avian tuberculin. The temperature records of the tests are given in Table 4.

TABLE 4.
TUBERCULIN TESTS ON HOGS FED AVIAN TUBERCLE BACILLI. AVIAN TUBERCULIN.

Pre-Injection Temperatures	Hog 1	Hog 2	Hog 3
8 A.M.	102.2	102.1
10 A.M.	102.2	102.0	104.1
12 M.	102.3	102.1	103.0
2 P.M.	102.3	102.6	102.7
4 P.M.	102.6	102.6	103.0
6 P.M.	103.0	102.6	103.3
8 P.M.	102.6	102.0	102.0
Post-Injection Temperatures			
6 A.M.	103.0	106.8	106.4
8 A.M.	103.2	106.8	104.5
10 A.M.	104.4	107.2	106.1
12 M.	105.4	105.6	105.4
2 P.M.	105.4	105.8	105.0
4 P.M.	105.8	105.3	104.7
6 P.M.	105.8	105.2	104.0

Hogs 1 and 2 were fed four times with contaminated food, beginning August 15 and ending October 7, 1912, and the tests were made three months later. The high thermal reaction persisted for 36 hours and was accompanied by a severe constitutional reaction and swelling at the point of inoculation. Hog 3 was injected with cultures about three months before the test was made. Neither the thermal nor constitutional reaction was as marked in this case.

The two hogs fed have grown rapidly and show no evidence

¹ Circular 201, Bureau of Animal Industry, U.S. Dept. of Agriculture, 1912.

of a progressive type of the disease. We purpose to keep them for a longer period.

As a check on the test, two healthy hogs were injected with avian tuberculin. No thermal or constitutional reaction resulted.

Relationship of the avian tubercle bacillus to the mammalian types.

—Most of the writers on avian tuberculosis have considered the question of the identity of the avian bacillus and the organisms from cattle and man. The usual conclusion has been that the avian organism represents a variety, caused by the adaptation of the organism to its environment, and that both the avian and mammalian are to be classed as one kind. It seems to the writers that there is very little ground for such an assertion. Culturally the avian organism is quite different from the mammalian types, differentiating itself by the rapidity and profuseness of growth and by the moist growth easily emulsified. The pigment development is marked and the growth at the bottom of the liquid media is certainly very different from the ordinary tubercle bacillus. It stains more easily and retains the stain less tenaciously than the mammalian types. Those who have held that all belong to one kind, based their conclusions largely on the similarity of the immunologic reactions.

Recent work indicates that the acidfast bacilli represent a group with many properties in common and that there is more or less interchangeability in their immunity reactions. Wills¹ studied the tubercle bacillus, the leprosy, timothy grass, urine and blind worm organisms by means of the complement fixation test. He found that the serum of an animal infected with tubercle bacilli would give a reaction when any of the above-mentioned organisms were used as antigen in the complement fixation test. He believes that all the acidfast organisms have definite substances, common to all, and that these bodies are concerned in the production of antibodies. He found a quantitative not a qualitative difference in the organisms employed. Thwort² studied by means of the agglutination and complement fixation reaction, the relation of the organism causing Johne's disease or chronic dysentery in

¹ *Centralbl. f. Bakteriol.*, I, Orig., 1911, 61, p. 37.

² *Centralbl. f. Bakteriol.*, I, Orig., 1912, 66, p. 316.

cattle, to other acidfast organisms. Animals were inoculated with human, bovine, and avian tubercle bacilli, with Johne's bacillus and with *B. Phlei*, a non-pathogenic organism. The result of the work showed the specificity of the tests to be small. A serum that gave a positive result with its homologous organism was usually positive with the others.

Little has been done by us along similar lines but the results have been confirmatory of those of Wills and of Thwort. The hogs to which avian tissues or pure cultures were fed have been tested with tuberculin made with mammalian cultures. The first two tested gave no reaction whatever. Hogs 1 and 2 in Table 4 were retested 80 days later using ordinary tuberculin. The results of the test are given in Table 5.

TABLE 5.
TUBERCULIN TESTS ON HOGS FED AVIAN TUBERCLE BACILLI. ORDINARY
TUBERCULIN.

Pre-Injection Temperatures	Hog 1	Hog 2
10 A.M.	103.6	103.0
12 M.	102.5	102.2
2 P.M.	102.4	102.4
4 P.M.	102.6	102.3
6 P.M.	103.0	102.7
8 P.M.	102.6	101.8
Post-Injection Temperatures		
6 A.M.	102.4	105.8
8 A.M.	103.4	103.8
10 A.M.	104.5	104.3
12 M.	103.8	103.6
2 P.M.	103.1	102.6
4 P.M.	103.6	103.2
6 P.M.	103.5	102.3

It is to be noted that the thermal reaction was much less than in the previous test made with avian tuberculin and that the fever was of short duration. No swelling at the point of injection or constitutional reaction was noted. We have been unable to kill tubercular hens by the injection of ordinary tuberculin. As high as 6 c.c. of concentrated tuberculin injected into the peritoneal cavity of a hen, later shown to have generalized tuberculosis, produced but a slight effect. Guinea-pigs shown by postmortem examination to have extensive lesions produced by avian bacilli were not killed by 2 c.c. of a tuberculin, 0.1 c.c. of which was sufficient

to kill guinea-pigs infected with mammalian tubercle culture. Reeser¹ concluded from his work that tuberculin made with avian cultures was useless in testing cattle. It was suggested by O. Bang that avian tuberculin could be used as a diagnostic agent for Johne's disease. In some cases the results of the postmortem examination has agreed with the test, in others not.

Many trials have been made to infect hens by feeding sputum containing tubercle bacilli. A few have reported successful results, but the great majority of trials have given negative results. In our work a fowl was fed a mixture of meal and sputum 25 times between June 23 and October 7. The sputa used were from various sources. Two other fowls were fed like material 20 times during a period of three months. The results in all cases were negative. In a number of the outbreaks brought to our attention it had been thought by the veterinarian that the cause was sputum to which the birds may have had access, since there were cases of tuberculosis in the families.

It seems quite probable that the acidfast bacilli represent a great natural group, that the great majority are saprophytic forms, occurring in the animal body, while a few are pathogenic, the most prominent being the mammalian tubercle bacillus, the leprosy organism and Johne's bacillus and that the avian organism bears the same relation to the true tubercle bacillus as does the leprosy organism and Johne's bacillus. As is well known some of the saprophytic forms may produce lesions in guinea-pigs that can not be differentiated from the true tubercle by a macroscopic or microscopic examination; especially is this true when they are introduced with a considerable amount of foreign matter such as butter fat.

It is of course possible that the avian organism may be capable of producing lesions, if not a more serious form of the disease in man. There is some evidence to indicate this, but it remains certain that the great importance of the disease is as an economic factor in the poultry industry. This factor is destined to become of greater importance unless the poultrymen recognize more than at present the necessity of purchasing birds and eggs from known healthy flocks.

¹*Centralbl. f. Bakteriöl.*, I, Orig., 1908, 46, p. 159.

SUMMARY.

1. Avian tuberculosis is widespread in the United States and Canada. Undoubtedly it is increasing rapidly in extent.

2. Avian tuberculosis is primarily a disease of the abdominal cavity. The liver was tubercular in 97 per cent of the cases examined, the spleen in 93 and the intestines in 60 per cent.

3. It is probable that the diseased bird is the important factor in the transmission of the disease from flock to flock. Eggs may also be a factor of some importance.

4. It seems probable that the avian tubercle bacillus is not a true tubercle bacillus, but rather bears the same relation to the bovine and human tubercle bacilli as do the organisms of leprosy and of Johne's disease. The acidfast bacilli represent a great natural group.

THE QUINCY (ILLINOIS) TYPHOID EPIDEMIC.*

EDWIN O. JORDAN AND ERNEST E. IRONS.

The city of Quincy is situated on the Mississippi River in the southwest part of Illinois about 100 miles above St. Louis. The population is nearly 37,000 (1900, 36,252; 1910, 36,587). In January, 1913, a sudden outbreak of typhoid fever occurred, involving approximately 200 bed cases. At the request of the city authorities an investigation of the epidemic was made by the writers early in February.

The usual record-cards were employed in the study of the outbreak, and full data regarding each patient were obtained wherever possible. In Quincy, as indeed in many American cities with 25,000-50,000 population, typhoid fever has not been a reportable disease, and at the beginning of our inquiry official records of cases were not available. Through the cordial co-operation of the Quincy physicians, however, a fairly complete list of bed cases was soon at our disposal.

There seems to have been little typhoid fever in Quincy between October 1 and December 25, 1912. We were able to obtain a history of several cases scattered along singly on the following dates: October 1, 7, 14, November 1, 24, December 1, 14, 15, and 18. On December 27 and 28, five cases developed, and from that time to about the first of February, new cases appeared daily. In all, we learned of 202 definite cases occurring in Quincy between December 27, 1912, and March 1, 1913.¹ We were able to secure quite complete information regarding 177 of this number. Except in a very few instances the patients had not been away from Quincy for at least 30 days before the onset of their illness. The cases comprised 99 males and 103 females. The age distribution was as follows:

Years	Cases
0-5.....	8
6-15.....	37
16-30.....	76
31-45.....	25
45 and over.....	12
No information.....	<u>44</u>
	202

* Received for publication June 9, 1913.

¹ In addition we learned of three out-of-town cases, one fatal, attributable to infection in Quincy.

The approximate date at which the disease developed was obtained in 153 cases. The method of tabulation was the same as that used in our study of the Rockford typhoid epidemic in 1912.¹ So far as could be determined the date of onset as shown by the earliest symptoms was as follows, tabulating cases in six-day periods:

	Period	Cases
January	1-6.....	11
"	7-12.....	29
"	13-18.....	42
"	19-24.....	35
"	25-30.....	29
"	31-February 5.....	7

This time distribution indicated the latter part of December and the first days of January as the probable period of maximum infection.

At Quincy as at Rockford² it was frequently difficult to determine the date of onset with any degree of exactness, owing to the attacks of typhoid fever having been preceded by an attack of gastro-enteritis. This complication is not uncommon in water-borne typhoid outbreaks. In some cases the enteritis is sufficiently severe to require the calling of a physician, and the patient remains more or less ill until the development of typical typhoid symptoms, it may be two or three weeks later. In others the attack of enteritis is slight and of short duration and the early typhoidal symptoms are attributed by the patient and his family to a simple recurrence of the original trouble. In the latter class of cases, a physician may not be called until the patient is in the second or third week of typhoid fever; in the former, a physician may be in attendance from the very beginning of the gastro-enteritis, two weeks or more before typical typhoid symptoms appear.

For these reasons some comment on the clinical features of epidemic typhoid seems worth while. In our studies of typhoid epidemics we have met with many cases in which the diagnosis was called in question because the disease presented clinical features which, though well recognized and often described, departed from the usual type of the disease. We were frequently met, too, with

¹ *Jour. Infect. Dis.*, 1912, 11, p. 21.

² *Loc. cit.*

the objection that if the typhoid fever in a community was due to an infection of the general public water supply, typhoid fever should appear in a larger proportion of the population than was found to be the case.

It is of course recognized that, of those individuals in a community who are exposed to typhoid fever through the infection of water, the number who suffer from typhoid fever may be relatively small. Thus in the Rockford epidemic, due to a sudden and temporary pollution of the water supply by sewage, the probable number of cases of initial enteritis was over 10,000 in a population of 50,000, but careful search for cases of typhoid revealed only about 200 cases. Out of a total of 46 persons residing in one block, 44 drank water within a few hours after the contamination occurred; 40 of these 44 persons drank sufficient water to produce gastrointestinal disturbance, in some instances, mild with little or no diarrhea, in others, very severe with nausea, vomiting, purging, and marked prostration, and yet only one person of the 44 suffered from typhoid fever later. It is of interest that this person did not suffer from the initial enteritis. It might be assumed that the sharp enteritis with copious evacuations was the means of ridding the body of typhoid bacilli before they were able to gain a foothold, but among the typhoid cases about which we were able to obtain information, approximately 90 per cent had suffered from some gastro-intestinal disturbance within a few hours after drinking the infected water. They were then free for the most part from symptoms until the onset of typhoid prodromes some days later.

In those persons in whom typhoid fever follows the ingestion of typhoid bacilli, the severity of the disease may vary within wide limits, and if the presence of an epidemic is not recognized, many mild cases pass without the diagnosis of typhoid being made. In both the Quincy and the Rockford epidemics we met with certain mild cases in which the fever was very slight, with moderate malaise, and headache, and in which a positive diagnosis was made only from examination of the blood or confirmed by the finding of *B. typhosus* in the stools. Other cases were seen in which the fever was moderate in degree (100–101° F.) with a duration of but 10 or 12 days and with practically no prostration nor malaise. In

some of these there was definite enlargement of the spleen and sometimes a profuse crop of rose spots. In the absence of a recognized epidemic some of these patients would not have consulted a physician, but would have attributed their indisposition to "colds," errors in diet, and the like.

Typhoid fever in the aged is particularly likely to be atypical, and pneumonia is well recognized as a frequent complication. In the Quincy epidemic, as will be noted later, there were an unusual number of cases diagnosed "influenza," "pneumonia," and "intestinal gripe" during the period of the epidemic, in an institution containing some 1,500 persons over 60 years of age. In no case was a clinical diagnosis of typhoid fever made, nor were the symptoms of illness of such a character to suggest such a diagnosis. Yet the water supply of this institution and of the city at large were the same, and the relatively high death-rate from the above diseases as compared with that of corresponding months in previous years in this institution, occurred at the same time as did the period of initial enteritis followed by typhoid in the city. It is of course probable that many of these cases of "influenza" were infections due to the polluted water ingested at the outset of the period of polluted water supply. A high death-rate, however, from pneumonia and influenza and severe gastro-intestinal disturbance continued long after the enteritis in the city had ceased; so that it seems probable that certain of these deaths were due to typhoid infection although not recognized as such.

Another factor that tends to obscure the diagnosis of typhoid fever in such epidemics is the recurrent character of many of the cases of the initial enteritis. In the Rockford epidemic the initial pollution of the water supply began and ceased abruptly and the maximum degree of pollution lasted only a few hours. A very large proportion of the population suffered from enteritis within 48 hours after the beginning of the pollution of the water. In the majority of the cases the enteritis subsided within a few days to a week. In about one-fourth of the cases, however, there were recurrences of diarrheal symptoms at intervals of three to five days with intervening periods of relative freedom from gastro-intestinal disturbance; as many as three or four of such recurrences were

noted in some cases. So, when the typhoid cases began to develop about two weeks after the initial infection, there were still some cases of recurrent enteritis in the practice of many of the physicians. Some physicians recognized clearly the presence of an epidemic and that some of the cases were typhoid fever and others recurrent enteritis. In the minds of others, however, the occurrence of the two types of the disease was very confusing, and, because the typhoid patients did not present the severe and typical forms of typhoid fever, the diagnosis of "abdominal grippe," "influenza," etc., was made in both forms of illness.

As has been pointed out in both these epidemics, we had to deal with two types of infection: the initial infection due to the pollution of the water supply with sewage, followed within ten days or two weeks by an epidemic of typhoid. It is undoubtedly true that in many instances the initial enteritis modified the development of subsequent typhoid fever, but quite apart from this influence the variation in the symptomatology in the typhoid cases has been much greater, with more mild and scarcely recognizable cases on the one hand and more severely toxic cases with high fever and delirium presenting the typical typhoid state on the other, than has been the case in epidemics originating in an infected milk supply.¹ The relative mildness and greater uniformity in type in milk epidemics of typhoid fever have been pointed out by other observers, but this difference has been so striking in the series of epidemics which we have studied that the point has seemed worthy of reiteration.

There were 16 deaths from typhoid fever in Quincy during the period covered by our inquiry. The deaths occurred on the following days: January 20, 25, 30, 31, February 4, 8 (2), 9, 10, 12, 13, 20, 21, 26, March 8, 13. Distribution of deaths according to age and sex was as follows:

Sex	Age	Cases	Deaths	Percentage
Male.....	0-10	30	2	5
Female.....	0-10	29	3	10
Male.....	20+	41	9	22
Female.....	20+	49	2	4

¹ Jordan and Irons, *Jour. Amer. Med. Assn.*, 1912, 63, p. 169.

At Quincy as at Rockford¹ the case-mortality among adult males was considerably higher than among males under 20.

As will appear presently, typhoid infection probably occurred in Quincy to a greater or less degree for at least two weeks, possibly for a longer period, so that it was not possible to make any sharp distinction between primary and secondary cases. There were in all: 177 one-case families; 9 two-case families; 3 three-case families. In seven cases the evidence of contact infection was practically conclusive and in at least as many more it was highly probable.

In an epidemic presenting the features described, and occurring in winter, fly-borne infection may be excluded. Personal contact, although responsible for some cases, could not cause so explosive and widespread an outbreak as this.

Data were collected so far as possible concerning the character of the milk supply. The records showed that 55 different milk-dealers supplied families in which typhoid had developed, that 15 patients had used condensed milk only, five had used a particular brand of canned cream, and eight had used no milk of any sort. In no instance so far as ascertained did the number of cases on any particular milk route bear a disproportionate ratio to the number of customers on that route. There was hence no reason to believe that the milk supply was responsible for the outbreak.

On the other hand, it was found that in all those cases regarding which information could be obtained, the city water was used constantly or occasionally. This factor was not lacking in any instance we investigated. In a number of families using well or cistern or bottled water, or boiled city water, all the members were exempt save those that drank city water at school or at their place of business.

The events preceding the typhoid outbreak are also suggestive of water infection. From about December 20 to the 5th or 6th of January many cases of the so-called "intestinal grippe" or "winter cholera" occurred in Quincy. According to information kindly furnished us by many physicians, these cases of "winter cholera" were very numerous and quite uniformly distributed through the

¹ *Op. cit.*, p. 23.

city. About December 28-January 4, this affliction was so widespread that it was generally commented on in the Quincy newspapers. During the same period the appearance of city water as drawn from the taps was such as to excite criticism in the daily press. One paper declared (December 31) that "boiling the water is much better than suffering all the various forms of stomach trouble which the doctors report in Quincy." And again: "Rarely if ever before have there been so many cases of the plague." Another paper (January 4) speaks of "nine out of every ten people in the city" as being affected with the "intestinal gripe."

This outbreak of enteritis was followed in from 10-20 days by a number of cases of typhoid fever. Allowing for the usual period of incubation of typhoid fever, this brings the date when the typhoid infection took place in exactly the same period with the enteritis infection.

It is thus clear that two outbreaks of disease occurred in Quincy in December, January and February—one, the enteritis with a short period of incubation, and the other, typhoid fever, following after a longer interval. This is no novel or unusual occurrence. Precisely similar outbreaks have been observed in Mankato, Minn.,¹ Rockford, Ill.,² and other places. An outbreak of gastro-enteritis and typhoid fever due to drinking-water obtained from the Mississippi River by excursionists on the steamer "G. W. Hill" occurred in July and August, 1912, and has been described in *Public Health Report No. 104* of the U.S. Public Health Service.

Conditions at the Soldiers' and Sailors' Home in Quincy with about 1,500 residents were particularly interesting. The average age of the residents is 71, the youngest being 60. From information kindly furnished by Dr. Ehle and others it appears that, while there were no cases of diagnosed typhoid fever in the period in question, there were hundreds of cases of diarrhea in December and particularly in early January. The advanced age of the patients undoubtedly influenced the course of the disease and also the diagnosis. It is worth noting that in January, 1913, seven deaths were recorded from "influenza," "enteritis," and "acute gastritis,"

¹ *Jour. Infect. Dis.*, 1911, 9, p. 710.

² *Ibid.*, 1912, 11, p. 21.

whereas practically no deaths had been recorded from these causes immediately prior to this outbreak or at this period in previous years.

There is reason to believe that the public water supply has played in the past a large part in the causation of typhoid in Quincy. While it is unfortunate that there has been practically no registration of typhoid cases, the board of health possesses excellent mortuary records. From a compilation made from them by Mr. W. R. Gelston, the average number of typhoid deaths reported annually per 100,000 population was as follows:

1870-1879.....	132
1885-1889.....	105
1890-1899.....	63 ¹
1900-1909.....	36

During the past four years the rates have been as follows:

Year	Deaths	Death-Rate per 100,000
1909.....	16	44
1910.....	10*	27
1911.....	8	22
1912.....	6	16

* Including one death from "paratyphoid fever."

The seasonal distribution of deaths is especially significant. In the four years, 1909-12, the deaths in 3-month periods are as follows:

December-February.....	14
March-May.....	11
June-August.....	7
September-November.....	8

These figures show a larger number of deaths in the winter and early spring months than in summer and autumn. This is a reversal of the usual seasonal distribution observed in cities with an uncontaminated water supply where, as is well known, typhoid is more prevalent in late summer and early autumn than at other times of the year. The excessive number of typhoid deaths in Quincy in winter and spring is evidence that some part has been played in the past four years by water infection. It may be noted especially that in 1912 there were only two deaths recorded between

¹ Filter plant built in 1891.

May 1 and December 31—the smallest number of deaths reported in a similar period during the four years in question. The reason for this will appear presently.

All the evidence obtainable from a study of the cases pointed to the responsibility of the public water supply: the explosive character of the outbreak; the age distribution of the cases; the absence of evidence implicating the milk supply or any other article of food; the coincidence between the widespread outbreak of “winter cholera” and the date when the typhoid infection must have occurred; the distribution of cases in correspondence with the density of population and the location of the water mains, and the fact that in the past four years typhoid fever has been more prevalent in Quincy in winter and spring than in summer and autumn.

The conditions surrounding the Quincy water supply may be briefly described.¹ The water is obtained from the Mississippi River through an intake pipe extending about 1,400 feet from the suction well at the pumping-station to a point in the Mississippi River a short distance south and west of the point of land marking the entrance to Quincy Bay (see diagram). “The intake pipe is 30” in diameter and with the exception of some 75’ or 80’ of iron pipe extending from the suction well, it is made up of wooden staves with bands around the staves to hold them in place. It ends in an intake crib weighted down to the bottom of the river with stone. In the crib is a cast iron pipe in a vertical position, which rises to a height sufficient to prevent the intake pipe from filling up with sand.” The dangerous position of the intake pipe may be noted on the accompanying diagram. The Broadway sewer, one of the largest of the main city sewers, empties into the river two blocks north of the pumping-station. During the early part of February, 1913, we observed that the flow of the warm water from this sewer was sufficient to keep unfrozen a stretch of the river extending some 20 or more feet from the shore. This flow from the sewer passes directly over the intake pipe, and the old wooden intake pipe is thus bathed a good deal of the time with fresh sewage. In case even small leaks exist in the pipe, the danger is obvious. In May, 1912, a leak in the intake pipe due to a broken or bent stave was

¹ The description is in part taken from personal observation, in part from a report, *On the Plant of the Citizens Water Works Co.*, made to the City Council of Quincy by Mr. C. A. Jennings in February, 1912.

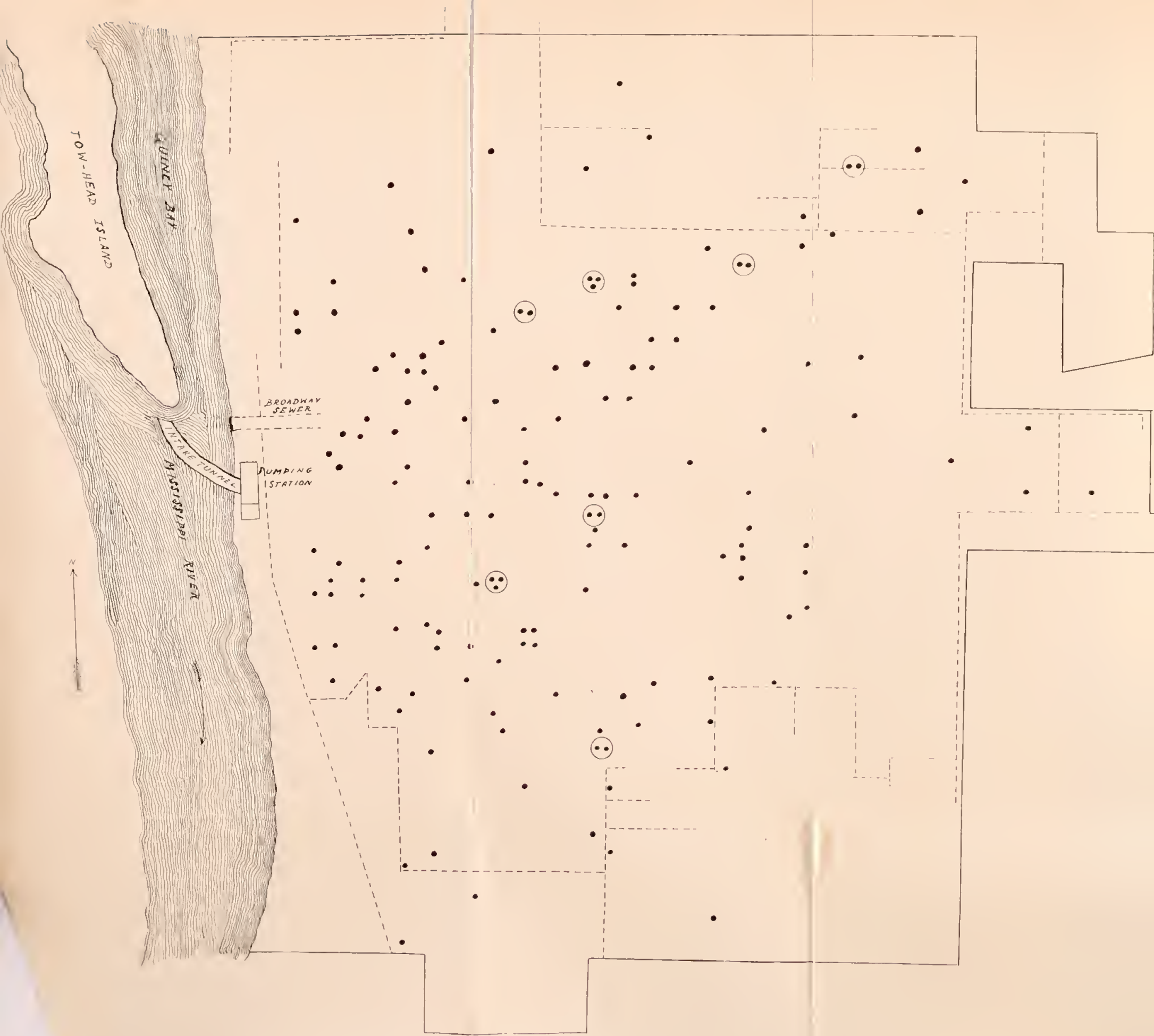


FIG. 1.—Diagram showing location of water works and distribution of typhoid cases, Quincy, Illinois. Full lines represent city limits. Broken line represents the approximate boundaries of the public water supply. Each black dot shows the location of a case of typhoid.

repaired by a diver employed for the purpose. Whether the pipe is now bacteriologically tight or not seems to be uncertain. At the time of our visit to Quincy it was impracticable to test the pipes for leaks owing to the ice on the river. Some bacterial observations made by Gelston in 1911 (cited in Jennings' report) and by Jennings in 1912 indicated an increase in the number of bacteria between the intake crib and the suction well. Whether the water at the intake crib is liable at times to sewage contamination from the Broadway sewer is a matter which would require a special and detailed investigation to determine. It seems possible, however, that a rapid change in the river level might cause deflection of some of the sewage toward the intake. It may be added also that the sewage from the Soldiers' Home and from some other places enters Quincy Bay above the point mentioned. The actual extent to which the intake is exposed to danger from these contaminating sources has never been fully investigated.

The water flows by gravity into the intake pipe, then by gravity into the suction well. There is no pipe directly connecting the intake pipe and the pump suction. The water discharges from the intake pipe into the suction well, the water level in it being the same as that in the river while the low-duty pump is not operating. From the suction well the water is pumped to a settling-basin which is far too small for efficiency, since it provides for a subsidence period of only little more than an hour. Sulfate of aluminum is used as a coagulant. The water then flows from the basin to a series of filters and thence to the high-duty pump. The surplus of water not drawn from the mains passes into a storage reservoir on high land to the northeast of the city.

The filters, 14 in number, are of the O. H. Jewell type and were installed about 20 years ago. They are circular wooden tanks, 12 inches in diameter, and are arranged in the filter house in two rows of five each and one row of four tanks. These filters are of the mechanical or gravity type and were designed to operate at a normal rate of filtration of about 125,000,000 gallons of water per acre of sand area per day of 24 hours. Each filter has an area of 113.1 square feet and at the normal rate of filtration will produce 13,504 gallons per hour or 324,104 gallons per 24 hours, no allowance

being made for time out of service while being washed or cleaned and prepared for operation after cleaning. It has been known for some years that the bacterial efficiency of these filters was low.¹ Jennings has shown that "the average percentage removal of bacteria for the period October, 1911, to February 9, 1912, inclusive, by the filters was only 86.2 per cent, with a minimum of 54.6 per cent and a maximum removal of 98.5 per cent." In view of this very unsatisfactory condition of the filtration plant, treatment of the water with bleaching powder was suggested as a safeguard. Bleach was accordingly added to the water in various amounts on the following dates:

Year				
1909	April	14-April	27 inclusive	
	May	4-June	9	"
	June	24-June	25	"
	July	6-August	5	"
	August	24-August	27	"
	October	11-November	8	"
	November	30-December	1	"
1910	February	14-February	15	"
	March	10-March	21	"
	March	23-May	26	"
1912	March	25-December	31	"
1913	January	3 to date		

There is ground for believing that the use of the calcium hypochlorite has been efficacious in some degree in preventing typhoid fever. The year 1912 during which the hypochlorite treatment was used for about nine months, had the lowest typhoid death-rate recorded in Quincy. From May 1 to December 15, 1912, only one death from typhoid fever was reported, a record unequaled in the sanitary history of the city. The coincidence is worth noting also that the next longest period of continuous hypochlorite treatment, March 23 to May 26, 1910, is followed by four months, April, May, June, and July, of complete exemption from typhoid fever deaths.

There is no doubt that the raw river water entering the intake well of the pumping-station is at times rich in bacteria. An examination in May, 1911, showed an average of 20,520 per cubic centi-

¹ W. R. Gelston, "Bleaching Powder as an Adjunct to Filtration at Quincy, Ill.," *Proc. Second Meeting of Illinois Water Supply Association*, 1910, p. 193.

meter, and Jennings found as many as 174,000 on February 7, 1912. Through the kindness of Mr. Gelston, we were able to examine the data of analyses made by him in 1912-13. The following table (Table 1) gives the average and range for four months.

TABLE 1.
1 C.C. COLONY COUNT (AGAR, 37° C.)

	INTAKE WELL			SETTLING BASIN			CLEAR WELL OR HIGH-PRESSURE PUMP		
	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum
1912 Oct.	87,900	1,000,000	1,700	16,000	100,000	100	470	4,000	12
Nov.	6,900	33,750	630	5,200	30,000	190	40	208	8
Dec.	2,000	9,950	250	1,500	3,250	240	140	402	21
1913 Jan.	6,400	112,500	105	1,800	50,000	110	350	702	20

GAS-PRODUCING BACTERIA 1 C.C. (DEXTROSE BROTH).

	INTAKE WELL	SETTLING BASIN	CLEAR WELL OR HIGH- PRESSURE PUMP	HYPOCHLORITE AVAILABLE CHLORIN. PTS. PER MILLION		
	Percentage Positive	Percentage Positive	Percentage Positive	Average	Maximum	Minimum
1912 Oct.	39	88	18	0.44	0.58	0.31
Nov.	68	82	33	0.44	0.60	0.18
Dec.	74	90	76	0.15	0.30	0.07
1913 Jan.	93*	79*	29*	0.44	0.96	0.00

* Lactose bile.

It will be noted: (1) that the raw water has at times a very high bacterial content; (2) that gas-producing bacteria are abundant; (3) that the percentage reduction both of bacteria growing on agar at 37° and of gas-producing bacteria was much less in December than in the other months; and (4) that the average amount of hypochlorite added to the water in December was much lower than in any other month. It should be observed especially that so far as hypochlorite dosage is concerned, the first few days of January belong with the December period. No bleach at all was added on January 1 and 2 and but 0.25 and 0.35 on January 3 and 4, respectively. Water samples from the high-pressure pump on the first five days of January showed the presence of gas-producers in all tests (1 c.c.) and a percentage reduction compared with the intake well on the same days of only 61 (January 3) and 62 (January 1)

per cent. On January 3, 702 colonies growing on agar at 37° C. were obtained in the water from the pressure pump.

Through a large part of the month of December, therefore, the amount of hypochlorite added to the water was not sufficient to bring about the degree of bacterial reduction usually considered requisite in treatment of water of this character. Many bacteria from the originally dangerously contaminated water were apparently able to pass through unscathed into the mains during this period.

The few bacterial examinations¹ that we were able to make during our stay at Quincy, February 3-13, showed that the water in the intake well during that period had a high bacterial content—140 to 665 colonies per cubic centimeter at 37° and 235 to 10,440 at 20°. Colon bacilli were found in every one of twenty $\frac{1}{2}$ c.c. samples examined February 10. During the same period the bacterial content of water samples collected from the high-pressure pump and from taps in various parts of the city ranged from 11 to 55 at 37° and 16 to 101 at 20°. Gas-producing bacteria were present in the proportion of 10 in 50 c.c. of the water (lactose broth). During this period the hypochlorite added to the water averaged 0.54 with a minimum of 0.40 (available chlorin, pts. per million).

SUMMARY AND CONCLUSIONS.

1. The Quincy typhoid epidemic occurring in January and February, 1913, included about 200 bed cases—a case-rate of about 1 in 180 of the population.

2. No evidence could be found that milk supply was in any degree concerned in the outbreak; fly infection and some other factors could be definitely excluded.

3. The epidemiological features of the outbreak pointed to water supply infection.

4. The Quincy water supply is taken from the Mississippi River and is dangerously exposed to contamination with fresh sewage from one of the city's own sewers.

5. Bacterial examination of water samples from the intake well

¹ The water examinations were made for us by Mr. A. H. Hixson, who also aided us in securing data respecting some of the typhoid fever cases.

of the Quincy Water Works has given in the past and still gives unmistakable evidence of gross contamination.

6. The present rapid filter system is entirely inadequate to free this raw water from dangerous bacteria.

7. There is evidence that the bleaching powder which was added to the water before its entrance into the mains from March 25 to December 1, 1913, prevented water-borne typhoid fever from reaching its usual prevalence in Quincy during this period.

8. The amount of bleach added in December, 1912, and the first few days of January, 1913, was much below the average for the preceding eight months. This reduction in the percentage of hypochlorite permitted sewage bacteria to pass through unharmed into the water mains.

9. The period during which an insufficient amount of hypochlorite was used corresponds with the date when the maximum typhoid fever infection occurred and also with the date of an extensive outbreak of gastro-enteritis.

10. The resumption early in January of adequate hypochlorite treatment was followed by the immediate subsiding of the epidemic.

THE IDENTITY OF *ENTAMEBA HISTOLYTICA* AND *ENTAMEBA TETRAGENA*, WITH OBSERVATIONS UPON THE MORPHOLOGY AND LIFE CYCLE OF *ENTAMEBA HISTOLYTICA*.*

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(WITH PLATES I AND 2.)

During the past three years a great deal of study has been devoted to the parasitic amebae of man, especially to the species that have been described as causing dysentery, and considerable advance has been made in our knowledge of these organisms. Among the subjects investigated the possible identity of *Entameba histolytica* and *Entameba tetragena*, the species most often described as associated with amebic dysentery, has attracted much attention, and the impression has been steadily gaining ground that these organisms, generally considered as being distinct species, are in reality identical.

Until quite recently I have considered *E. histolytica* and *E. tetragena* as distinct species, but the study of certain material, to be hereafter described, has convinced me that *E. tetragena* is identical with *E. histolytica*; that Schaudinn and, afterward, myself, incorrectly interpreted certain changes occurring in this entameba as reproduction by "budding" or spore formation; and that to Viereck, whose description was amplified by Hartmann, belongs the credit of first correctly describing the life cycle of *E. histolytica*, although he named the entameba which he described as a new species, *E. tetragena*.

In order thoroughly to understand the exact position of the question of the identity of these entamebae at the present time, it is necessary to review the literature dealing with this particular point *in extenso*, and in doing so I shall quote at length from the more important papers, in order that each investigator's position in the matter may be clearly understood.

* Received for publication May 5, 1913.

In 1903, Schaudinn¹ published a paper upon the amebae of the intestinal tract of man, in which he accepted Casagrandi's and Barbagallo's division of amebae into two genera, *Ameba* and *Entameba*, the latter genus including all of the parasitic species, and demonstrated that in the intestine of man there occur two distinct species, one common to healthy individuals and those suffering from diseases other than dysentery, while the other was found only in patients suffering from amebic dysentery. To the former he gave the name *Entameba coli*, and to the latter, the name *Entameba histolytica*. He based his differentiation of these species upon marked differences in morphology and in their life cycle.

Schaudinn described *E. coli* as an entameba showing but little distinction between the ecto- and endoplasm; having a distinct nucleus possessing a rather thick nuclear membrane and a well marked karyosome; reproducing by simple division; schizogony, with the formation of eight amebulae, and by the formation of cysts containing eight daughter nuclei.

He described *E. histolytica* as an entameba showing a marked distinction between the ecto- and endoplasm; a nucleus characterized by a delicate nuclear membrane and a minute karyosome, the nucleus being generally invisible in the living organism; and as reproducing by simple division and by a process of "budding" or spore formation. The latter process he described as consisting of the budding off, from the periphery of the organism, of minute portions of cytoplasm containing nuclear chromatin, the buds later developing a resistant membrane, and only undergoing further development when they reached the intestinal canal of a suitable host. Schaudinn states that he produced typical dysentery in two cats by feeding them material containing these spores and that typical entamebae appeared in the feces of the experimental animals.

I was able to confirm Schaudinn's observations in 1905² and my results will be referred to later in this communication.

Viereck,³ in 1906, described what he considered to be a new species of entameba in a patient suffering from dysentery contracted in Africa, and called it *E. tetragena*. The same parasite was described almost simultaneously by Hartmann,⁴ and since then this species has been found to have a worldwide distribution and the observations of these authorities regarding it have been confirmed by many investigators.

E. tetragena was described by Viereck and Hartmann as an entameba showing a marked distinction between the ecto- and endoplasm; having a distinct nucleus possessing a rather thick nuclear membrane; a large karyosome containing generally a distinct centriole; while it reproduced by simple division and by the formation of cysts containing four daughter nuclei, thus distinguishing it, according to these authors, from *E. coli*, which forms cysts containing eight daughter nuclei, and from *E. histolytica*, which was said to reproduce by "budding" or spore formation.

In 1909, Hartmann⁵ published a monograph upon *E. histolytica* in which he confirmed Schaudinn's description of this species and pictured forms which he interpreted as "budding forms," or reproduction by spore formation. In this publication he says: "Since the publication of Schaudinn, *Entameba histolytica* has been accurately described only by Craig, Werner, and myself, and Schaudinn's description thereby confirmed."

¹ *Arb. a.d.k. Gsndhtsamte.*, 1903, 19, p. 547.

⁴ *Ibid.*, 1908, 5, p. 117.

² *Am. Med.*, 1905, 9, pp. 854, 897, and 937.

⁵ *Arch. f. Protistenk.*, 1909, 18, p. 207.

³ *Arch. f. Schiff's- u. Tropen-Hyg.*, 1907, 11, p. 1.

It is thus evident that at this time Hartmann was convinced that *E. histolytica* was a distinct species and that the description given by Schaudinn had been confirmed by himself, as well as others.

In Hartmann's excellent chapter entitled "Die Dysenterie-Amöben" in S. von Prowazek's *Handbuch der Pathogenen Protozoen* (1911), he casts doubt upon the accuracy of his former description of *E. histolytica* and states that he evidently mistook degenerative forms of *E. tetragena* for certain stages in the life cycle of *E. histolytica*, and that, after studying very carefully all of Schaudinn's material, he has come to the conclusion that in only one case, an infection contracted in China, can he confirm the description of *E. histolytica* as given by Schaudinn. He calls attention to the probability that many of the so-called "budding" forms were really degenerative in character and that similar forms occur in infections with *E. tetragena*.

In a still later monograph upon *E. tetragena*, published in 1912,¹ Hartmann describes in detail the character of the nucleus in this species, a structure he considers of the greatest importance in differentiating this species from other entamebae, and states that he considers *E. histolytica* as a rather doubtful species. He believes thus, because *E. tetragena* is the only entameba he has encountered in a considerable number of cases, and because of his failure to find any evidence of reproduction by "budding" or spore formation in this dysentery entameba.

At the present writing, Hartmann's position is apparently as follows: He does not absolutely deny the existence of *E. histolytica* as a distinct species but inclines strongly to the belief that it is identical with *E. tetragena*.

In 1911, Major Whitmore, U.S. Army Medical Corps, published a paper² detailing the results of his study in Hartmann's laboratory of material from cases of amebic dysentery contracted in the Philippine Islands and Saigon. He confirms the presence in Manila of *E. coli*, first noted by myself in soldiers returning from the Philippines, but finds that the entamebae present in all of his material from dysenteric cases was *E. tetragena*, not a single case of infection with *E. histolytica* being observed. He therefore concludes that the common dysentery entameba of Manila is *E. tetragena* and that infections with *E. histolytica* must be very uncommon.

In a later paper³ Whitmore accepts the specific status of *E. histolytica* and *E. tetragena* but, in speaking of the encystment of the latter species he says:

"Under the conditions that cause *tetragena* to encyst, there are always a great many degenerating amebae in which the nucleus is in all stages of disintegration, and great care is necessary in order not to misinterpret such findings. Again, at this time, the chromatin is passing off from the nucleus into the plasma to form chromidia, and such an appearance may easily be mistaken for the nuclear changes which take place in *histolytica* preparatory to budding."

Major Whitmore states⁴ that he believes *E. tetragena* and *E. histolytica* to be identical and that it would appear that the name *tetragena* should be abandoned in favor of *histolytica*.

¹ *Arch. f. Protistenk.*, 1912, 24, p. 163.

² *Ibid.*, 1911, 23, p. 70.

³ *Arch. Int. Med.*, 1912, 4, p. 515.

⁴ Personal communication.

The identity of these two species was discussed very fully in a paper by Walker,¹ who studied the subject in Manila. This observer was the first definitely to state that *E. tetragena* is identical with *E. histolytica*, and his description of the morphology and life cycle of the latter parasite I believe to be more nearly correct than that given by any other investigator. Practically all that has been accomplished since the publication of his paper has been confirmatory of his work and at the present time I believe that his conception of the specific status of *E. histolytica* and *E. tetragena* is the correct one. Walker's paper is of especial interest because he dealt with a large amount of material in Manila where infection with entamebae is so frequently observed.

After discussing the amebae found in the water supply of Manila and those which can be cultivated, he gives his results in the study of the amebae found in the intestine of man. He concludes that all of the amebae which can be cultivated are free living species, belonging to the genus *Ameba*—a conclusion previously reached by the writer and confirmed by Hartmann, Whitmore, and others—and that the amebae found in the intestine of man are strictly parasitic and belong to the genus *Entameba*, as shown by the same investigators. He also confirms the presence of the harmless entameba, *E. coli*, in Manila, but recognizes only one species of pathogenic entameba, i.e., *E. histolytica*, which he thinks includes the entameba described by Viereck and Hartmann as *E. tetragena*, and that described by Elmassian as *E. minuta*. He says, after describing *E. coli*: "The other species includes the *histolytica* variety of Schaudinn, the *tetragena* variety of Viereck and Hartmann, and probably the *minuta* variety of Elmassian. It is characterized by the hyaline appearance, indistinct nucleus, and active motility in the living entameba; by the feebly staining, reticulated cytoplasm, and by the relative paucity of chromatin which is arranged either as a barely perceptible layer about the inner surface of the nuclear membrane, with or without a few fragments scattered in the nuclear network (*histolytica* variety), or as a more extensive but loose, granular peripheral layer and a loose central karyosome (*tetragena* variety), with transitions between these two varieties in the stained entamebae; and especially by the development of cysts containing four nuclei. This species is found only in the stools, pus, or tissues of amebic dysentery, amebic abscesses, or of cases having a history of amebic dysentery, and is probably a pathogenic species. According to the law of priority this species should bear the name *Entamoeba histolytica* Schaudinn."

Walker is thus the first to recognize that the nuclear structure of *E. histolytica* varies greatly under different conditions, at times presenting the typical *histolytica* type of structure, described by Schaudinn and myself; while at others it presents the typical *tetragena* type, described by Viereck and Hartmann.

In his paper two cases are described in which, during the active stage of dysentery, the entamebae were all of the *histolytica* type, and no cysts could be found, but when the symptoms disappeared and the stools became normal, the characteristic *tetragena* cysts appeared. He rightly says:

"During the active phase of amebic dysentery only the trophozoites of these entamebae are present in the stools. . . . When the acute symptoms in untreated

¹ *Philippine Jour. Sc.*, 1911, 6, p. 379.

dysentery have passed and the stools of the patient are becoming normal, the trophozoites become smaller, less actively motile, and more rounded in the resting forms, and the chromatin becomes more abundant in the nucleus. These changes are preparatory to the development of cysts. . . . Finally, encysted forms containing four nuclei appear which may persist for an indefinite period or until the patient suffers from an exacerbation of the acute symptoms."

Walker was unable to find any forms which he could interpret as showing reproduction by "budding" or spore formation. His description of the two types of nuclei observed in *E. histolytica* is so accurate that it is here reproduced:

"Two varieties in the distribution of the chromatin can be distinguished. In the one the chromatin is arranged as a barely perceptible layer about the inner surface of the nuclear membrane, with or without a minute karyosome, or a few scattered fragments in the nuclear network. This variety corresponds with the *histolytica* species of Schaudinn. In the second variety the chromatin is rather more abundant and is arranged in part as a loose granular layer, that frequently shows radial projections, about the inner surface of the nuclear membrane; and in part as a loose central karyosome which, in its most typical form, consists of a minute centriole surrounded by an achromatic halo that is bounded by a circle of chromatic granules. This variety corresponds with the *tetragena* species of Viereck and Hartmann."

Walker found that entamebae showing the *histolytica* type of nucleus were observed more frequently in Manila than those showing the *tetragena* type, thus confirming my own observations in this respect.

He concludes his valuable paper by stating that he recognizes two species of entamebae as being present in his material: a harmless species, *E. coli*, and a presumably pathogenic species, *E. histolytica*, which includes the species described by Viereck and Hartmann, and named *E. tetragena*; and that it is possible to differentiate these species by means of the microscope. His paper is accompanied by photomicrographs illustrating the variations in the type of nucleus observed in *E. histolytica*.

Several valuable papers, published by Darling upon the entamebae observed in the Canal Zone, will now be considered.

In a report¹ of the Board of Health Laboratory, Canal Zone, published in 1912, he states that investigations demonstrate that *E. histolytica* "is the chief, if not the sole, pathogenic entameba in this region."

Regarding *E. tetragena* he notes that this species has been found twice and says: "This species of entameba is not frequently encountered and it is not regarded as being a very pathogenic type."

¹ Report of Board of Health Lab., Dept. Sanitation, Isthmian Canal Commission, 1912, p. 42.

As showing how liable one is to err if one depends largely upon nuclear structure in the differentiation of species of entamebae, Darling's results are of interest and value, for in this paper he concludes from the study of the structure of the nucleus that the common entameba of Panama is *E. histolytica*. He says:

"In order to determine positively, if possible, the type of entameba that has actually invaded the tissues of man in this region, a variety of pathological material has been examined with regard to this point, paying particular attention to the morphology of the nucleus of the invading entameba as stained by hematoxylin, there being structural differences between the nuclei of *E. histolytica*, *E. tetragena*, and *E. coli* when stained carefully with hematoxylin. In only one instance out of 22 individuals that had died of entamebic dysentery was an entameba found in the tissues resembling *E. tetragena*—though not positively identified as such. In all other instances the entamebae correspond with *E. histolytica*."

The author closes this contribution by stating that "*E. histolytica* is the chief, if not the sole, pathogenic entameba in this region," but in a paper published about six months later¹ Darling reverses his conclusions regarding *E. histolytica* and says:

"From a careful study of entamebae obtained from clinical cases and autopsy material in this region, I am not only of the opinion that the only pathogenic entameba of Panama is *E. tetragena*, but I am in agreement with Hartmann and Doflein that the entameba, usually described as *E. histolytica* from cases of dysentery, is *E. tetragena*."

Darling bases this statement upon the occurrence of cysts containing four nuclei in his material, the absence of budding forms described by Schaudinn and myself, and the fact that, in infected kittens, vegetative forms were observed that showed the *histolytica* type of nucleus:

"Many trophozoites were found which had the staining and morphological characters not only of *E. tetragena*, but of *E. histolytica* and *E. nipponensis*. These variations in the morphology of the nucleus depend upon variable amounts of chromatin and variations in the distribution of the same within the nuclear membrane." Regarding the occurrence of cysts in his cases, he says: "The likelihood of finding cysts in any given case depends on several circumstances. In progressively fatal cases in man and animals in which the individual succumbs to infection, there may at times be seen, in addition to the large trophozoites, some small forms containing idiochromidia, but I have never detected cysts in these cases. In early acute cases, in first infections, for example, where the lesions were presumably superficial, and where active medication by mouth had been administered, I failed to find developmental forms."

In January, 1913, Darling, in another paper,² definitely states his belief that *E. tetragena* is the only pathogenic entameba:

¹ *Jour. Trop. Med.*, 1912, 15, p. 257.

² *Arch. Int. Med.*, 1913, 11, p. 1.

"If we make a careful study of the entamebae in our cases of dysentery and liver abscess, using a technic which brings out, as well as possible, the morphological features of the nucleus of trophozoites and in the cysts, we can be of no other opinion than that there is but one pathogenic entameba and that one is *E. tetragena*." In this paper Darling states that treatment appears to have a great influence in preventing the development of cysts, and, as will be seen later, this undoubtedly explains why cysts have not been observed in many infections with *E. histolytica*.

In still later contributions¹ Darling describes the occurrence, in kittens infected with *E. tetragena*, of forms identical with *E. histolytica*, especially the forms answering to the description of "budding" or spore formation, as given by Schaudinn and others. His description of these forms is as follows:²

"The material showing the budding forms was in the fourth remove, at the time of the death of the kittens, that is, when the strain had become mature or senile, reduced in size until it resembled *E. minuta*, when chromidia had appeared in every trophozoite, and a few cysts had appeared. In well fixed hematoxylin preparations the peculiar changes noted in some of the trophozoites were as follows:

"1. The appearance of buds of different sizes on the periphery of the trophozoites in question. These buds were usually clear and free from chromidia. Some, however, contained chromidia and not infrequently a bud contained the nucleus.

"2. The extrusion of chromidia, nuclei, and portions of cytoplasm. Chromidia was seen apparently being extruded. The nucleus always took up an extreme peripheral position in the cytoplasm, was frequently attached by a slim pedicle of cytoplasm and later detached. Round portions of the cytoplasm [buds] were extruded."

Darling regards these forms as degenerative in character and believes that his observations "establish a correlation between the findings of Schaudinn and Craig on the one hand, and those of Viereck, Werner, and Hartmann on the other."

It will be noted that Darling found these "budding" forms in wet-fixed, hematoxylin-stained preparations, thus confirming my own results and disproving the statement of some authorities that such appearances are found only in preparation air-dried and stained with some modification of the Romanowsky stain, and that they were due to the staining or fixing process.

At the present writing Darling evidently believes that *E. tetragena* is the only pathogenic entameba and that *E. histolytica* was described from degenerative forms of *tetragena*. While his observations are largely confirmatory of those of Walker, already noted, he has thrown considerable light upon the occurrence of the

¹ *Science*, 1913, 26, p. 16; *Jour. Am. Med. Assn.*, 1913, 60, p. 1220.

² *Op. cit.*

so-called "budding" and has shown that such forms occur only under certain conditions.

In a valuable paper, published in 1912, Wenyon¹ gives the results of experimental research upon the production of dysentery in cats with *E. histolytica* and *E. coli*. Regarding the identity of *histolytica* and *tetragena* he says:

"It seems highly probable that the pathogenic ameba with which Schaudinn worked was the common pathogenic form and that he failed to recognize the cysts of Viereck with their four nuclei and that the small spores he described were other structures unconnected with the amebae. If this position be adopted it comes about that the ameba of Viereck, *E. tetragena*, is none other than *E. histolytica* of Schaudinn, and that Viereck regarded it as a distinct species because he had discovered the true life history, which, of course, differed from the erroneous one given by Schaudinn. Hence, provided Schaudinn was right in naming the pathogenic ameba *E. histolytica*, the name *E. tetragena* is no longer required. The common pathogenic ameba of man is still *E. histolytica*, but the life history is not that described by Schaudinn, but that discovered by Viereck."

Wenyon calls attention to the fact that the characteristic cysts occur only in a small proportion of cases of amebic dysentery, and that he has not been able to confirm the occurrence of reproduction by "budding" in *E. histolytica*. Wenyon was successful in producing dysentery and liver abscess in cats with this entameba but all of his experiments with *E. coli*, the harmless entameba, resulted negatively. His successful case of liver infection in a kitten was long antedated by that of the writer, who produced liver abscess in a kitten with *E. histolytica* as far back as 1905.²

The latest contribution upon the subject is that of James, who has studied a large amount of material upon the Isthmus of Panama and in the Canal Zone. I have had the pleasure of examining many of his preparations. In a paper published in April, 1913,³ he states that he is undecided regarding the identity of *E. histolytica* and *E. tetragena*, and thus describes the types of entamebae observed in his cases:

"In the active vegetative stage, associated with dysentery, those entamebae which exhibited the characteristics of *histolytica* did not go on to cyst formation, but persisted unchanged, even when the stools were semisolid, except for the formation, in some instances, of the so-called chromidia. Two of these cases were carefully followed for a period of three weeks. On the other hand, in all but one instance, the infections in

¹ *Jour. Trop. Med.*, 1912, 2, p. 27.

² *Op. cit.*

³ *New York Med. Jour.*, 1913, 97, p. 702.

which the entamebae were of the characteristic *tetragena* type went on to cyst formation, with a marked remission, even an apparent cure of the symptoms, and without medication."

Regarding the value of nuclear structure in the differentiation of entamebae, James says:

"I do not find that nuclear morphology, in specimens from stools with pus and blood, is a definite factor in the determination of species. Even with the most careful differentiation, many of the organisms in undoubted *tetragena* infections show nuclei that are exactly similar to those described for *histolytica*; and more than once, in infections in which the entamebae were nearly all of the *histolytica* type, and when there was no cyst formation, typical *tetragena* nuclei were observed. . . . Also, I have not, at any time, been able to observe the 'spore formation' described by Schaudinn and others."

The literature reviewed presents the present opinions regarding the identity of *E. histolytica* and *E. tetragena*, and shows very clearly, I think, that every investigator mentioned has really been studying the same parasite but variously interpreting certain authorities upon appearances noted during different stages in its life history. A careful perusal of the literature leads one almost inevitably to the conclusion that we have really been working with only one species of entameba, but our imperfect understanding of the whole life cycle, and the unwarranted emphasis laid by certain authorities upon nuclear structure as a means of differentiating species, has prevented the recognition of the identity of these parasites by many observers.

PERSONAL OBSERVATIONS.

At the time of the appearance of Schaudinn's paper upon the entamebae, I was so fortunate as to be in charge of the laboratories of the U.S. Army General Hospital at San Francisco where hundreds of patients suffering from entamebic dysentery were being received from the Philippines, and the material for the study of entamebae was almost unlimited. As a result of the study of these parasites I published in 1905¹ a paper confirming Schaudinn's findings, and this paper was followed by another in 1908² amplifying and confirming the observations already reported. In these papers I recognized the existence of *E. coli*, the harmless entameba, and *E. histolytica*, the pathogenic species, and confirmed Schaudinn's description of reproduction by "budding" or spore formation in the latter species.

¹ *Op. cit.*

² *Jour. Infect. Dis.*, 1908, 5, p. 324.

In 1911 I reported the occurrence of *E. tetragena* in the Philippine Islands, Panama, and the United States,¹ and recognized this organism as a distinct species, basing this conclusion upon the occurrence of cysts containing four nuclei during one stage in the life history of the parasite. At this time I stated that I had undoubtedly overlooked this species in the material examined at San Francisco, confusing it with both *E. coli* and *E. histolytica*. In other contributions, published in 1911² and 1912,³ I stated my belief that there are three species of entamebae parasitic in man, i.e., *E. coli*, *E. histolytica*, and *E. tetragena*, and in a paper read before the Fifteenth International Congress for Hygiene and Demography, in September, 1912,⁴ I stated my position regarding the identity of *histolytica* and *tetragena* as follows:

"At the present time some authorities believe that this species [*histolytica*] is identical with *Entameba tetragena*, but the point is far from proved, and until it can be shown that the process of reproduction by gemmation and spore formation, described by Schaudinn as characteristic of *Entameba histolytica*, and confirmed by myself in many cases of dysentery, occurs also in *Entameba tetragena*, and that cases of dysentery occur which never present the characteristic four nucleated cyst at any stage of the disease process, in infections with *tetragena*, I am forced to consider the two species as distinct, despite the fact that the nuclear structure of *Entameba tetragena*, during certain stages of development, resembles very closely that of *Entameba histolytica*."

As the result of the study of material since the above paper was written I have become convinced that these two so-called species are really identical, and that the process of reproduction by spore formation, described by Schaudinn, and confirmed by myself and others, is actually a degenerative change occurring especially in cases under treatment. Furthermore, I am satisfied that in this class of cases, and in cases presenting active symptoms of dysentery, *E. histolytica* does not undergo encystment, in the vast majority of instances, but reproduces only by simple division, thus explaining the absence of cysts in most of the material that I have studied.

The character of the cases studied at San Francisco must be considered in any discussion of the species of entamebae associated with them. All patients observed there had been returned to the

¹ *Arch. Int. Med.*, 1911, 7, p. 362.

² *The Parasitic Amoebae of Man*, Philadelphia, 1911, p. 9.

³ *Jour. Med. Research*, 1912, 26, p. 1.

⁴ *Am. Jour. Med. Sc.*, 1913, 145, p. 83.

United States from the Philippines because of the severity and intractable nature of the entamebic infections from which they were suffering; all were under treatment by means of rectal injections of quinine and other substances destructive to the entamebae; and nearly all presented active symptoms of the infection. Despite daily treatment a considerable proportion of these infections terminated fatally, and those who apparently recovered frequently relapsed, and in many the relapse proved fatal. In this class of patients the entamebae did not go on to cyst formation, and a certain, though small proportion, showed the so-called budding forms of Schaudinn. In those cases that eventually recovered, cysts were sometimes observed but were interpreted at that time as the cysts of *E. coli*, for it should be remembered that *E. tetragena* had not then been described. As the entamebae associated with these cases corresponded to Schaudinn's description of *E. histolytica*, I had no hesitation in confirming his description. As later researches have shown that in such cases cysts are seldom observed in *tetragena* infections, and as Darling has recently shown that, in infections in kittens, where the entamebae have multiplied rapidly, forms are observed similar to those described by Schaudinn, as well as the absence of cysts, I feel sure that I misinterpreted degenerative changes for a reproductive process. The fact remains, however, that too little stress has been laid upon the occurrence, in just this class of cases, of entamebae presenting the typical nuclear structure of *E. histolytica*, for most of the writers upon *tetragena* have insisted that the nuclear structure was entirely distinct from that of *histolytica*. As a matter of fact, *histolytica* presents all variation in nuclear structure from that described as typical of it, to that described as typical of *tetragena*.

The material that I have recently studied consists of three cases of entamebic dysentery occurring in the Canal Zone, preparations from which Dr. W. M. James has kindly sent me, and one case at the Soldiers' Home in Washington, whose infection was contracted in the Philippine Islands. I am greatly indebted to Dr. James for sending me specimens from his cases, for the many preparations he forwarded contain examples of nearly every stage in the life history of *E. histolytica*. The following short history of each case is

abstracted from his description of the cases which accompanied the specimens:

Case 1.—Patient a Greek, aged 40 years. Two and one-half years in the Canal Zone. Admitted January 24, 1913, with fever and diarrhea. Blood showed tertian malarial plasmodia. Stool examination on January 25 showed numerous entamebae with very faintly visible nuclei, the nuclear membrane represented by a few granules of chromatin. In a few a centriole is seen, but there is no indication of a karyosome. Ectoplasm and endoplasm easily differentiated. Stained specimens showed the typical *histolytica* type of nucleus. The entamebae remained of this type until January 30, 1913, the patient being in bed during this time upon a milk diet. On January 30, the entamebae were decidedly smaller in size, the ectoplasm less marked, and the nuclei obscured. On January 30, the nuclei were still of the *histolytica* type, but upon February 2 the entamebae were much smaller, resembling *E. minuta*, and well defined nuclei were seen. The patient had meanwhile been placed upon a diet of eggs, potatoes, toast, etc. Quite a few four-nucleated cysts were found at this date. On February 3, the stools were semi-formed and cysts were numerous, sometimes three or four in a single field. The patient was then placed on the bismuth treatment and the entamebae all disappeared from the stools in two days.

Case 2.—Patient a Spaniard. Six years in the Canal Zone. Attacks of dysentery for two months previous to admission. Admitted January 25, 1913. Stool first examined January 26, 1913. Consistence semi-fluid. Blood and mucus present. Many entamebae. In this specimen "budding" was observed in many of the entamebae, which showed nuclei of the *histolytica* type. James states that he regards the "budding" as degenerative, for the stool was over two hours old and cold at the time of examination. On January 27, the stool again contained entamebae of the *histolytica* type. The patient was placed upon soft diet and from January 31 to February 5 the stools contained smaller entamebae and numerous typical *tetragena* cysts. (My own examination of these specimens showed that entameba of both the *histolytica* and *tetragena* type were present.)

Case 3.—A French boy, aged 19 years. History of dysentery for eight days. His stools contained pus and blood. A few entamebae were seen in the vegetative stage resembling *E. minuta*. (I found besides these a few organisms of the typical *histolytica* type.) On February 9, a few cysts were observed of the *tetragena* variety.

Regarding these cases Dr. James says:

"They [the preparations] are of particular interest in that they show what I take to be *histolytica* going on to encystment. This is either true, or there is a question of a double infection with disappearance of the *histolytica* forms. This latter, however, is rather improbable when one considers that the same process happened in three consecutive cases within a few days. Heretofore I have kept my patients under observation on liquid diet, but this time all three were put on soft diet, and in each instance encystment followed within a few days."

James calls attention to the fact that the entameba found in cases showing acute symptoms is almost invariably of the *histolytica* type, while the *tetragena* type is found in more chronic cases and in infections in cats. This agrees with my own observations and dis-

proves Hartmann's statement that entamebae showing the *histolytica* type of nucleus are degenerative forms. Anyone who has studied the entamebae occurring in acute attacks of dysentery knows that they almost invariably present nuclei of the *histolytica* type, and that the *tetragena* type of nucleus occurs most often in chronic infections and just before encystment. It is evident that both Viereck and Hartmann have studied chronic cases of dysentery or they would have recognized this fact.

The following case, occurring at the Soldiers' Home in Washington, has been under my observation for three years and is of interest because it demonstrates that *E. histolytica* undergoes encystment, and that such encystment may be delayed for a long period of time.

Case 4.—An American, aged about 36. Contracted dysentery in the Philippines in 1903. First examined in February, 1909, when he was having a relapse and very active symptoms of dysentery. At this time the stools contained many entamebae of the *histolytica* type and no cysts were observed, although the stools were examined repeatedly, even after apparent recovery occurred. Since that time the man has several times suffered from a severe relapse and always entamebae of the *histolytica* type were observed in the stools during the period of active symptoms. No cysts were observed until March, 1913, when, after the absence of symptoms for several months, and treatment with ipecac and quinine irrigations had been stopped for some time, an examination of the stools, after a dose of magnesium sulfate, showed numerous typical four-nucleated cysts, and entamebae of the *histolytica* and *tetragena* types, as well as "budding" forms. As a double infection could be practically eliminated in this case the evidence is almost conclusive that *E. tetragena* is identical with *E. histolytica* and, taken in conjunction with the cases of James, is conclusive to my mind.

In the vast amount of material I have had the opportunity of studying, covering over 1,000 cases of entamebic dysentery, the following groups of cases, as regards the character of the entamebae present, have been noted:

Group 1.—Cases in which nearly all the entamebae presented the typical *histolytica* nucleus, and that never went on to cyst formation, but not infrequently showed the "budding" forms described by Schaudinn in cases apparently recovering. These cases comprised the vast majority of those studied at San Francisco and it was upon this type of entameba that I based my confirmation of Schaudinn's observations. These cases presented marked symptoms and many of them terminated fatally. Practically all were under treatment at the time of observation.

Group 2.—Cases in which the entamebae presented both the *histolytica* and *tetragena* types of nucleus and a few cysts could be found after a long search. The entamebae presenting the *tetragena* type of nucleus varied greatly in size, and the cysts contained four daughter nuclei. Until recently I had interpreted these cysts as developmental forms of the cysts of *E. coli* when they occurred in association with vegetative forms of the *histolytica* type. “Budding” forms were sometimes observed and clinically the cases were of much milder type than those in Group 1.

Group 3.—Cases in which the entamebae present were of both the *histolytica* and *tetragena* types but in which the four-nucleated cysts were present in great numbers. Clinically these cases present few or no symptoms of dysentery; the stools were semi-formed, and no treatment was being administered.

Group 4.—Cases in which all the entamebae present were smaller in size than those observed in the other groups, presented most frequently the *tetragena* type of nucleus, or a type intermediate between that and the *histolytica* type, in that, while no large karyosome was present, the nuclear membrane was much thicker than in the large vegetative forms of *histolytica*, and large numbers of four-nucleated cysts were observed. Clinically these cases presented slight or no symptoms and were not receiving treatment.

Group 5.—Cases in which there were mixed infections with *E. coli*, and entamebae answering the description of *E. histolytica* or *E. tetragena*. Clinically these cases varied greatly, as would be expected.

An analysis of the findings in these various groups of patients leads inevitably to the following conclusions:

1. Entamebae presenting the *histolytica* type of nucleus are found most frequently in patients presenting the most severe symptoms of entamebic dysentery. This type of nucleus appears to be characteristic of *E. histolytica* when it is undergoing rapid multiplication by simple division, for no cysts are observed in these cases. That this type of nucleus is not degenerative, as claimed by Hartmann, is proven by the fact that such entamebae occur most frequently in the worst cases of acute dysentery, and even though treatment is being administered, the entamebae showing this type of

nucleus differ entirely in appearance from those showing real degenerative changes brought about by the treatment. In addition, typical dividing forms are generally observed, showing this same type of nucleus.

2. Entamebae presenting the typical *tetragena* type of nucleus occur most frequently in cases presenting slight dysenteric symptoms and this type of nucleus appears to be characteristic of *E. histolytica* when it is undergoing simple division preparatory to the formation of the small generation which produces cysts.

3. Entamebae reduced in size, and presenting generally the *tetragena* type of nucleus, although an intermediate type between this and *histolytica* is frequently observed, occur most frequently in cases of long duration (chronic dysentery), in which the symptoms are practically subsiding. This type is apparently characteristic of *E. histolytica* preparatory to the formation of cysts.

4. The four-nucleated cysts occur most frequently in cases which have apparently recovered and in which the stools are semi-formed or formed. These cysts are characteristic of *E. histolytica* but occur only in a very small proportion of cases, as shown by Wenyon,¹ Ornstein,² James,³ and others, and do not appear in acute infections, as shown by their absence in such infections in man and in fatal infections in kittens.

5. Entamebae presenting appearances that have been interpreted as reproduction by "budding" or spore formation occur most frequently in cases presenting subacute symptoms of dysentery. Such forms are associated with entamebae showing the *histolytica* type of nucleus and occur in cases in which the entamebae are undergoing rapid multiplication by simple division. Most of the patients were receiving treatment by rectal injections of quinine. Similar forms, much smaller in size, are also found in association with cysts and are not infrequently observed in patients who have apparently recovered. I believe that these forms are degenerative in nature and may be produced by exhaustion of the race due to rapid multiplication, as suggested by Darling, or by the effect of remedial agents, as quinine. Personally, I am of the opinion that both

¹ *Op. cit.*

² *Arch. f. Protistenk.*, 1913, 29, p. 78.

³ Personal communications.

causes may operate in producing these so-called "budding" forms of *E. histolytica*.

OBSERVATIONS UPON THE MORPHOLOGY AND LIFE CYCLE OF
E. histolytica.

In the light of our present knowledge it becomes necessary to reconstruct the life history of *E. histolytica* and to recognize that the nuclear structure of this species may vary, at different times in the life history, from that typical of *histolytica*, as described by Schaudinn, to that typical of *tetragena*, as described by Viereck and Hartmann. This fact was first clearly stated by Walker but the insistence of Hartmann and his followers upon the absolutely diagnostic structure of the nucleus in *E. tetragena* led to great confusion and delay in the recognition of the identity of this species with *E. histolytica*. Having been led astray myself because of relying too much upon morphology in the interpretation of certain phenomena occurring in the entamebae, I am convinced that too much stress has been laid upon morphological details in the differentiation of species and too little upon the study of the entire life cycle of these organisms. I agree thoroughly with James in his statement that nuclear morphology *alone* is not sufficient for the determination of species among the entamebae, for even in the hands of the most expert this method of determining species has failed not infrequently. While nuclear morphology is sufficient to differentiate the entamebae from the free living amebae of the genus *Ameba*, it is not sufficient for the determination of species within the genus *Entameba*. The nucleus of these parasites is constantly changing in morphology in answer to metabolic changes and differences in the environment. In addition, our fixing and staining methods are still far from perfect and undoubtedly produce more or less change in the appearance of the nucleus, as can be easily proven by varying the time of staining and amount of differentiating with the various solutions employed for this purpose. I am now referring to the most approved methods of wet-fixation and staining, for all of the observations recorded in this paper have been made upon wet-fixed and stained preparations.

From our present knowledge of the subject I believe that the

following description of the morphology and life history of *E. histolytica* is approximately correct. Briefly stated, the life history may be divided into the following stages: a vegetative stage of development, in which multiplication occurs rapidly by simple division; a pre-cystic stage in which multiplication occurs by simple division and the entamebae become markedly reduced in size; and a cystic stage, during which cysts containing four nuclei are developed. Of course, organisms are frequently encountered in any one of these stages, with the exception of the first, which belong to one of the other stages of development, but in the vast majority of our cases these definite stages may be observed if the conditions are favorable and the cases are followed for a long enough time. Cases that are acute or upon vigorous treatment will not show cysts, but if they become chronic and treatment is omitted, the pre-cystic and cystic stages of development can be demonstrated.

Vegetative stage of development.—It is in this stage of development that *E. histolytica* attains its largest size, sometimes exceeding 65 microns in its longest diameter, but averaging generally from 25 to 40 microns. In the living specimen there is a well marked distinction between the ecto- and endoplasm and the organism is actively ameboid. The nucleus may or may not be visible, but in most instances is invisible in acute cases of dysentery. As two distinct types of nuclei may occur during this stage of development, it is only when organisms presenting the *tetragena* type of nucleus are present that we are able to distinguish a nucleus in the living specimen. The cytoplasm contains bacteria, crystals, and in cases in which blood is present in the feces, one or more erythrocytes. Vacuoles are present but are not contractile.

In preparations wet-fixed and stained with iron hematoxylin or other hematoxylin stains, there is no distinction between the ecto- and endoplasm, the cytoplasm appearing coarsely granular or containing numerous small vacuoles or one or more large vacuoles. Two distinct types of nucleus are present in these vegetative entamebae, the so-called *histolytica* type and the *tetragena* type. In the former type the nuclear membrane is well stained and consists of a delicate, very thin membrane, upon the inner side of which a few minute chromatin granules may be observed. This membrane

often appears not over a line in thickness. A very small and delicate karyosome, called by some authors a centriole, often appearing simply as a minute dot of chromatin, is generally observed at or near the center of the nucleus. A centriole is not present within this karyosome.

A few minute dots or grains of chromatin may sometimes be seen lying within the nucleus between the karyosome and the nuclear membrane and traces of a linin network may sometimes be observed. The entire nucleus appears delicate and is often situated at the extreme periphery of the organism.

Reproduction by simple division is frequently observed in entamebae showing this type of nucleus, and in almost every specimen examples of this type of reproduction may be observed. In most instances the two nuclei produced by division are exactly similar in structure, and that structure is of the *histolytica* type, but rarely an entameba is seen containing one nucleus of the *histolytica* type and one of the *tetragena* type. Such an organism is illustrated in Fig. 6 and definitely proves that both types of nuclei are present in *E. histolytica*.

This type of nucleus is most frequently observed, as has been stated, in acute attacks of dysentery and is typical of most of the entamebae seen in the feces when severe symptoms are present. In cases showing less marked symptoms, or where the disease has become chronic, the vegetative entamebae generally show nuclei of the *tetragena* type. In this type the nuclear membrane is much thicker and better defined, while there is a comparatively large karyosome, situated at or near the center of the entameba, which contains a well marked centriole. The chromatin within the nucleus is much larger in amount than in the *histolytica* type, and is either scattered upon the inner side of the nuclear membrane and as minute, deeply stained masses upon the linin network between the membrane and the karyosome, or as larger, irregularly shaped masses upon the inner surface of the nuclear membrane. The karyosome is characteristic, consisting of a large, irregular or spherical mass of chromatin, often appearing connected with the nuclear membrane by delicate filaments. A centriole is often present consisting of a deeply stained, spherical dot of chromatin surrounded by a well

marked, unstained halo. Many variations are observed in the structure of this type of nucleus which are believed by Hartmann to be cyclical in character. The karyosome sometimes appears as a fine network inclosing an unstained area within which lies the centriole, while the outer border of the karyosome is formed of deeply staining dots of chromatin arranged in a circular manner. Again, the karyosome may stain evenly throughout with the exception of a clear, unstained halo around the centriole, or the halo may be absent, the centriole appearing as a deeply staining, almost black, mass within the karyosome. Rarely the entire karyosome is surrounded by an unstained area, the remainder of the nucleus presenting a dimly stained network having arranged upon it deeply stained dots of chromatin, or the chromatin may be arranged in bandlike masses near the nuclear membrane.

In many cases, if one searches carefully, intermediate types of nuclei between the *histolytica* and *tetragena* types may be seen, and a whole series of nuclei may be traced from that typical of *histolytica*, as described by Schaudinn, to that typical of the *tetragena* nucleus, described by Viereck and Hartmann.

In subacute cases of entamebic dysentery the so-called "budding" forms of *E. histolytica* may be found associated with these vegetative stages in the life history of the organism. These forms are most often found after the acute symptoms have lasted for some time and are just beginning to decline, generally as the result of treatment with rectal injections of various therapeutic agents. In other words, they are most often encountered when the race of entamebae has become weakened by rapid multiplication and by therapeutic measures. Organisms are observed in which the chromatin is being ejected from the nucleus and distributed in the cytoplasm. Others are noted in which the nucleus has disappeared and the entire cytoplasm is filled with masses of chromatin, while still others are observed in which these masses of chromatin are being "budded" off from the periphery of the parasite, surrounded by a small amount of cytoplasm. Many of these "buds" appear to develop a membrane around them and no structure can be distinguished. I am now convinced that all of these forms are degenerative in character and have nothing to do with any process of reproduction.

Pre-cystic stage of development.—In this stage of development the entamebae multiply by simple division, but become markedly reduced in size, resembling the entameba described by Elmassian as *E. minuta*. The majority of the organisms also show a nucleus which may be said to be intermediate in structure between that of the *histolytica* and *tetragena*, having a thick nuclear membrane and a small, solid appearing karyosome. While entamebae are found at this stage with nuclei like those of both the *histolytica* and *tetragena* types, most of the organisms show nuclei like that described. The most marked feature at this stage of development is the small size of the vegetative forms, and to this generation of the parasite, James and Darling have given the name "the small pre-cystic generation."

Most cases showing these forms will also show a few typical four-nucleated cysts if they are carefully searched for, although there may not be more than one or two found in a single preparation.

Cystic stage of development.—The cystic stage of development quickly follows the pre-cystic. In cases showing cysts the vegetative forms, just described as characteristic of the pre-cystic stage, may be present in very small numbers, but when cyst formation is complete these vegetative forms are generally absent.

The organisms which become encysted generally contain more or less chromatin in their cytoplasm and in both the pre-cystic and cystic stages of development forms are rarely observed which resemble the "budding" forms found most frequently in the vegetative stage but they are much smaller and are really degenerated pre cystic or cystic forms.

When the cyst is fully developed, the chromidia will be found collected in one or more spindle-shaped masses within the cyst, which stain deeply and can readily be distinguished from any nuclei that may be present. The cysts measure from 7 to 20 microns in diameter, the average being about 15 microns, and are spherical in shape. The smaller cysts exactly resemble those described as characteristic of *E. minuta*. A distinct membrane may rarely be observed surrounding the cyst in stained preparations. The cytoplasm stains uniformly, and vacuoles are absent unless the cyst is degenerating, while extraneous matter, such as bacteria, erythro-

cytes, etc., has been extruded before the formation of the cystic membrane. The nucleus prior to division resembles that found in the vegetative form in the pre-cystic stage, having a rather thick nuclear membrane and a small, but distinct karyosome (centriole, of some writers). The nucleus divides into two nuclei, reduced somewhat in size, which have a very definite nuclear membrane and a well defined karyosome, composed of a few minute granules or a solid mass of chromatin. These two nuclei divide, the result being a cyst containing four nuclei. At the final stage of division the nuclei are much reduced in size and each of the four possesses a well defined nuclear membrane thin in outline, and a minute karyosome arranged as a solid mass of chromatin somewhat irregular in shape, or as a minute collection of chromatin granules.

Many of the four-nucleated cysts do not show the large spindle-shaped masses of chromatin alluded to above, so that it is evident that these have nothing to do with the vital processes involved in the formation of the four nuclei.

This brief description of the forms of *E. histolytica* observed in the various stages of its life history covers very inadequately the many variations that occur in every stage, but gives as composite a picture of the morphology of this interesting and important parasite as we are able to give at present. It has been proven that the cystic stage is alone infective to cats, and thus it is evident that in human infections the patients who show cysts are the ones that are a menace to others. As pointed out by Walker, these patients are really "carriers" of entamebic dysentery and the greatest care should be taken in the disposal of their excreta.

Nomenclature and classification.—I believe that it is best, at the present time, to recognize but two species of entamebae as being parasitic in the intestine of man, *E. coli* and *E. histolytica*. I think that the researches of several investigators have proven that *E. tetragena* is identical with *E. histolytica*, and the other species that have been described have not been confirmed and are still of uncertain value.

If this classification be adopted, the name *tetragena* should be dropped, as it becomes merely a synonym of *histolytica*. The fact that Schaudinn incorrectly described a portion of the life history of

histolytica and overlooked the cystic stage of development does not vitiate the status of this parasite as a distinct species. All authorities are practically agreed upon the fact that Schaudinn differentiated a harmless from a pathogenic species of entameba, and it makes no difference, so far as the species are concerned, whether his entire description was correct or not. He did describe correctly the vegetative stage of the parasite as observed in the vast majority of cases of dysentery presenting severe symptoms, and distinguished this species from *E. coli*, the species he found in healthy individuals. Therefore the name *Entameba histolytica*, which he gave this pathogenic species, must, by the law of priority, be retained as the specific name, and the name *tetragena* ceases to have any specific value. This conception of the specific name depends, of course, upon the accuracy of the name Schaudinn employed. If, as some believe, the pathogenic Entameba should have been named *Entameba coli* (Lösch) or *Entameba dysenteriae* (Councilman and Lafleur), either of these names should replace *histolytica*. At any rate, the name *tetragena* has now no status as a specific name.

CONCLUSION.

In concluding the discussion of these entamebae I cannot refrain from calling attention to the misinterpretations which are rendered almost unavoidable when protozoölogists insist upon laying so much stress upon minute morphological details in so variable a structure as the nucleus, in the differentiation of species of entamebae. Hartmann's imperfect description of *E. histolytica* (*E. tetragena*) has confused almost every worker on these organisms, as his omission of the fact that the typical *histolytica* nucleus occurs in the most acute cases of dysentery, while the *tetragena* type of nucleus is found in milder cases, and his really absurd statement that the *histolytica* type of nucleus is a degenerative form has, of course, resulted in most authorities regarding *histolytica* and *tetragena* either as distinct species or that Schaudinn's description of *histolytica* was entirely erroneous.

In a recent communication received from Dr. W. M. James, of Panama, he agrees with me that *E. histolytica* and *E. tetragena* are identical and that the *histolytica* type of nucleus is found chiefly in acute and severe infections. His observations will be published later, but they confirm, in almost every detail, all that is published in this paper.

I desire to take this opportunity of acknowledging my indebtedness to Dr. James for the many excellent preparations he has sent me and for a great deal of valuable data upon the entamebae he has studied in the Canal Zone.

EXPLANATION OF PLATES 1 AND 2.

The photomicrographs¹ were taken with a No. 2 eyepiece and a one-twelfth inch immersion objective. All specimens were wet-fixed and stained with iron hematoxylin.

FIG. 1.—Vegetative form (trophozoite) of *E. histolytica*, showing typical *histolytica* type of nucleus. $\times 1300$.

FIG. 2.—Vegetative form of *E. histolytica*, showing a modified *tetragena* type of nucleus. $\times 1300$.

FIG. 3.—Vegetative forms of *E. histolytica*, showing *tetragena* type of nucleus. The karyosome is too deeply stained to show the centriole. $\times 1300$.

FIG. 4.—Vegetative form of *E. histolytica* undergoing simple division. The nucleus has just divided and the two new nuclei are of the *tetragena* type. $\times 1300$.

FIG. 5.—Vegetative form of *E. histolytica* undergoing simple division. The two new nuclei have separated and are of a modified *tetragena* type. $\times 1300$.

FIG. 6.—Vegetative form of *E. histolytica* undergoing simple division. This organism is of great interest as it shows one nucleus having a typical *histolytica* structure and one having just as typical a *tetragena* structure. $\times 1300$.

FIG. 7.—A so-called "budding" form of *E. histolytica*. This form has been misinterpreted as multiplication by spore formation. The chromatin is scattered throughout the cytoplasm and some of it is being budded off from the periphery of the parasite. $\times 1300$.

FIG. 8.—Pre-cystic vegetative form of *E. histolytica*. Note reduced size and intermediate type of nucleus, the karyosome being larger than in the large vegetative *histolytica* and smaller than in the *tetragena* type of nucleus. $\times 1200$.

FIG. 9.—*E. histolytica* just at the time of encystment, containing one nucleus similar in structure to that in Fig. 8. $\times 1150$.

FIG. 10.—Encysted form of *E. histolytica*, showing the primary division of the nucleus into two daughter nuclei. $\times 1150$.

FIG. 11.—Encysted form of *E. histolytica*, showing three daughter nuclei. The fourth nucleus, if ever present, has degenerated and is not visible. $\times 1150$.

FIG. 12.—Encysted form of *E. histolytica*, showing four daughter nuclei. This is the fully developed form of the cyst and is typical of this species. $\times 1150$.

FIGS. 13 AND 14.—Degenerated cystic forms of *E. histolytica*, showing the distribution of the chromatin in masses in the cytoplasm. These forms have also been misinterpreted as "budding" forms or reproduction by spore formation, by some authorities. $\times 1150$.

¹ I am indebted to Captain Arthur R. Christie for the photomicrographs here reproduced.

PLATE I.

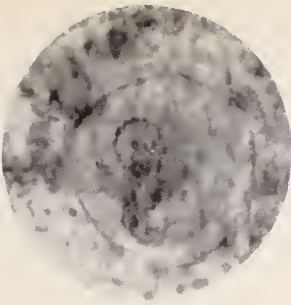


FIG. 1.

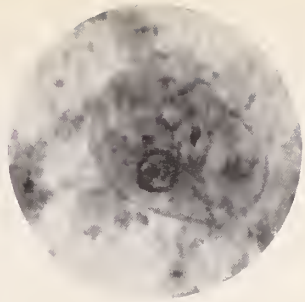


FIG. 2.

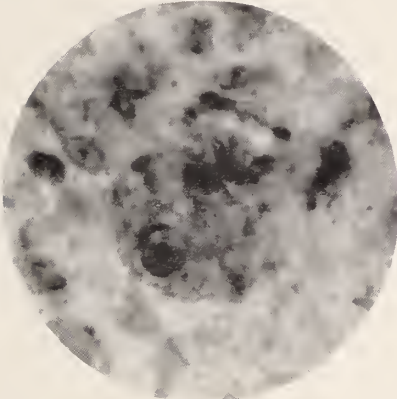


FIG. 3.

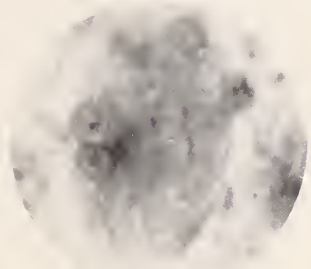


FIG. 4.

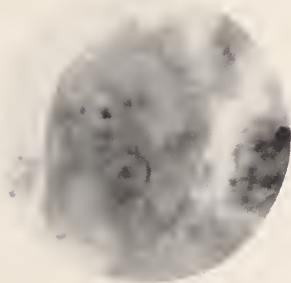


FIG. 5.

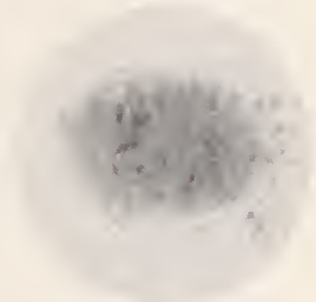


FIG. 6.

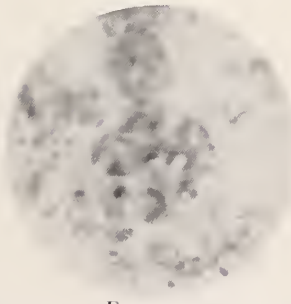


FIG. 7.



FIG. 8.

PLATE 2.

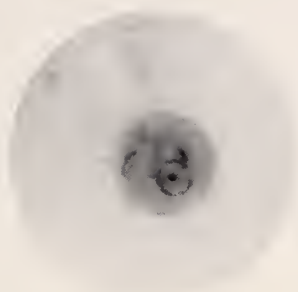


FIG. 9.

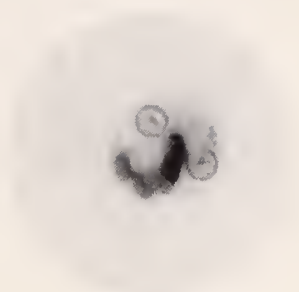


FIG. 10.

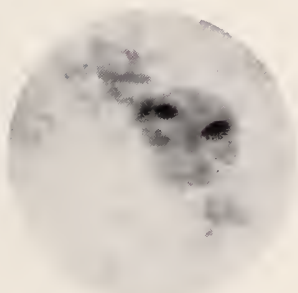


FIG. 11.

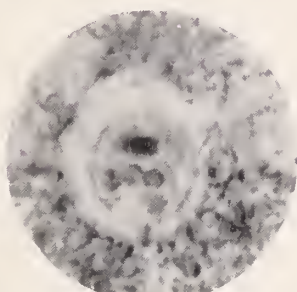


FIG. 12.

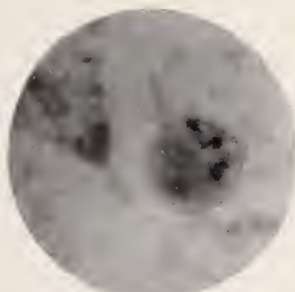


FIG. 13.

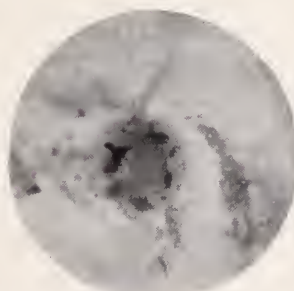


FIG. 14.

INVESTIGATIONS OF THE ETIOLOGY OF INFECTIOUS ABORTION OF MARES AND JENNETS IN KENTUCKY.*

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Probably no section of the United States has as high-priced horses as the Blue Grass region of Kentucky, the home of the thoroughbred, the standardbred and the saddle horse. It is no uncommon thing for the breeders of this section to pay thousands of dollars for individuals—even the hundred-thousand-dollar mark has been reached. Occasional outbreaks of infectious abortion among the mares are a great source of financial loss to the breeders of these horses. It has been estimated that Kentucky alone has sustained a loss running into the millions of dollars from the results of this disease. There have been seasons when as many as 70 per cent of the mares in some studs aborted, and occasionally a stud is reported where all the mares have slipped. This disease is not confined to Kentucky, for outbreaks have been noted in other states, as well as in Canada. According to Williams,¹ the disease first made its appearance in the United States in 1886, when it appeared in several states of the Mississippi Valley, in which region the breeding of horses had become a very important industry. He states that at that time the malady acquired a very extensive distribution and high degree of virulence.

In this part of the country, mares are bred soon after foaling. If the disease of infectious abortion is present in a stud of mares, most of the slips occur between November 1 and March 1. Close observers state that more abortions occur during the eighth month of pregnancy than at any other period. Mares carrying their foals ten and one-half months usually deliver them alive. We have examined some cases where the abortions occurred in the early stages of gestation. Usually a mare will abort a fetus well along

* Received for publication April 26, 1913.

¹ *Veterinary Obstetrics*, p. 478.

in gestation with little effort, and generally with no injurious sequelae. Occasionally an individual succumbs from metritis, after aborting.

The discharge from a mare after aborting is of a chocolate color. The veterinarians in this section of the country note a peculiar odor associated with the fetus and fetal membranes of a case of typical abortion. We have found this odor to be more pronounced in some cases than in others, and not only detected on the exterior of the fetus and in the internal organs, but sometimes on the after-birth also. The laymen usually speak of it as a "sour odor." Chemists at this station say it resembles an etheric odor.

The contents of the stomach of the fetus are acid to phenolphthalein at first, but upon standing soon turn to an alkalin reaction. The lungs show areas of hepatization. The spleen usually appears enlarged and covered with petechiae. The mesenteric lymphatic glands are often congested and the liver may show hemorrhagic areas on its surface.

Colts that come alive from a stud of mares affected with abortion are often rheumatic and have swollen joints. This malady is known among breeders of horses as "rheumatism" or "joint ill." Colts affected with "joint ill" appear very lame and walk with difficulty, while in some cases abscesses form at the joints. The hock, knee, ankle, or any joint may be affected, but the articulation at the hock is the usual seat of this trouble. The eyes may be involved, the sight becoming affected, and total blindness may result. In some cases, in a stud afflicted with contagious abortion, colts delivered at the full term of gestation, although they appear strong and healthy when foaled, suddenly sicken and die with a form of pneumonia, while others may become affected with white diarrhea, which usually terminates fatally. A large number of mares in a stud may abort during one year and nearly all deliver live healthy colts the next year.

Ostertag, of Germany, who has investigated the etiology of this disease among mares, found in the heart blood of the fetus, in the thoracic cavity, and in the intestinal tract a streptococcus which usually grew in pairs. He also secured this germ from the fetal membranes. This organism was gram-negative.

In 1890, Kilborne¹ found a short bacillus possessing pathogenic properties in the vagina of a mare which had recently aborted. This mare was one of a number that aborted in a large stud in Pennsylvania. Theobald Smith² made a study of this bacillus and



FIG. 1.—Mare and aborted fetus. Abortion caused experimentally by an intravenous injection of 2 c.c. of culture suspension of *B. abortus equinus* in physiological solution. Incubation period 10 days.

decided that morphologically and physiologically it resembled *B. cholera suis*. The characteristics of this bacillus, as it appeared on an agar slant, were a tendency to form wrinkles near the drop of

¹ Bull. No. 3, Bureau of Animal Industry, Washington, 1893, p. 49.

² *Ibid.*, 1893, p. 53.

condensation, and the membranous nature of its growth. A pure culture of this bacillus injected intravaginally into both a pregnant mare and cow produced a catarrhal discharge from the vagina for some time, but the mare delivered her foal and the cow her calf, alive.

Much speculation over the cause of the disease has been made by the breeders. The trouble is caused, according to some theories,



FIG. 2.—*Bacillus abortus equinus*. Plain agar streaked with heart blood of an aborted fetus of a mare. Culture 24 hours old. Actual size.

by the mare eating frost-bitten grass late in the fall, or early in the spring; by the flushing of the system due to eating too much succulent feed; by the presence of malodors, i.e., if a mare aborts in a field and the fetus and afterbirth are not removed at once and the place on the ground from which they were taken covered with dirt, the odor will of itself cause other mares to abort; by the running of mares on the same pasture year after year, or the feeding

of too much salt. Some breeders are of the opinion, however, that the trouble is due to a microbe.

No conclusive results having been obtained on the etiology of this disease, the Animal Husbandry Division of the Kentucky Agricultural Experiment Station feeling that this was a subject vital to the special breeding interests of Kentucky, equipped a laboratory for investigation along this line.

In April, 1912, a preliminary report of these investigations was made,¹ in which it was stated that a bacillus belonging to Subgroup II. of the Colon typhoid group, to which group *B. enteritidis* belongs, had been isolated from the afterbirth, the uterine exudate and internal organs of fetuses of mares and jennets infected with contagious abortion. This organism in most instances was found in abundance. Since that time, these investigations have been continued to determine whether or not this bacillus could be found in an outbreak of the disease in a subsequent year, and if the disease could be produced experimentally with this germ.

HISTORY OF THE STUDS.

Stud No. 1.—The disease existed in this stud in April, 1911. All five jennets in this stud aborted in quick succession.

Stud No. 2.—The disease existed among the mares in this stud in the fall and early winter of 1911; 10 out of 19 mares in one field aborted in a comparatively short time.

Stud No. 3.—History incomplete. This stud consisted of a very few mares. One abortion reported.

Stud No. 4.—Sixteen out of 30 mares of this stud, in one field, aborted during the late fall of 1912.

Stud No. 5.—Twenty-eight mares aborted in different fields on this farm during the late fall and winter of 1912 and the early winter of 1913.

Stud No. 6.—History incomplete. A number of mares aborted on this farm during the late fall and winter of 1912.

Stud No. 1, Case No. 1.—On June 24, 1911, a fetus and afterbirth were obtained from the fifth and last jennet to abort in this stud. The fetus arrived at the laboratory within half an hour after it was dropped. The fetus was in the eleventh month of gestation. Streaks of blood and contents of the following organs were made on numerous plates of plain agar, standardized neutral to phenolphthalein: heart, liver, spleen, kidneys, stomach, lymphatics, large intestines, small intestines, uterus, and ovaries. Each organ was seared and afterward opened with a sterile lance and one or two loopfuls of the contents streaked on plain agar, as in making streak dilutions. These plates were then incubated 24 hours at 37° C. and examined, with the result that a

¹ E. S. Good, *Ann. Rept. Ky. Agri. Exper. Sta.*, 1912, p. 225.

pure culture in abundance was found on all the plates. Platings from the afterbirth revealed the same organism in abundance. After making an extended study of this organism it was placed in Subgroup II, or Intermediate group of the Colon typhoid group, to which subgroup belong *B. enteritidis* and *B. cholera suis*.

Stud No. 2, Case No. 1.—On November 1, 1911, a fetus covered with the fetal membranes from a mare aborting in the eighth month of gestation was brought to the laboratory. The same organism was isolated from all the organs of this fetus and from the afterbirth, as was obtained from the fetus and afterbirth of the aborting jennet in Stud No. 1.

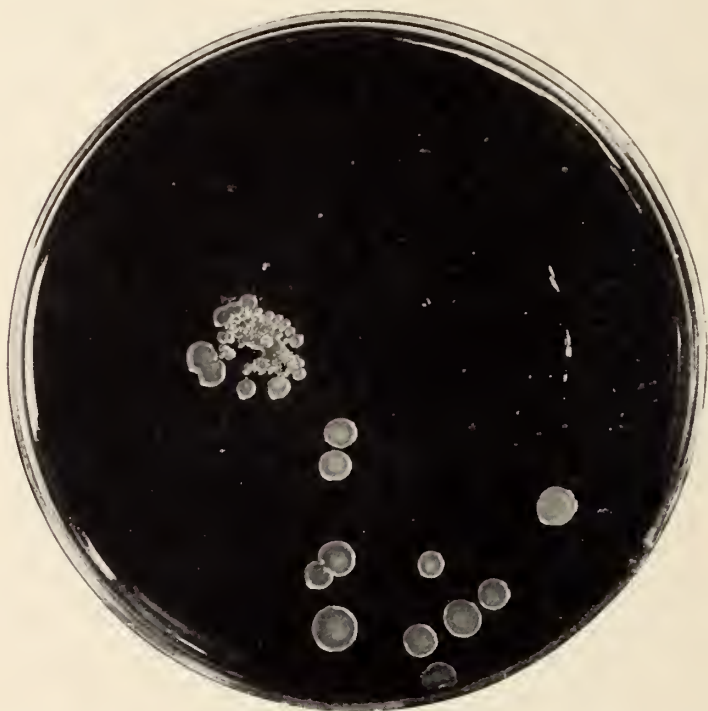


FIG. 3.—Colonies of *Bacillus abortus equinus* on plain agar. Four days old. Petri dish originally streaked with the uterine exudate of an aborting mare. Actual size.

Stud No. 2, Case No. 2.—This mare aborted in the 212th day of gestation. The fetus and afterbirth were destroyed by burning. Material was taken from the uterus the next day and plated, with the result that the same organism was obtained as has been previously mentioned.

Stud No. 2, Cases No. 3 and 4.—On November 13, 1911, we learned that two mares in this stud had aborted and the fetuses and afterbirths been burned. On November 16, Dr. J. T. Shannon of Lexington, at our request, visited this place and took some of the contents of the uteruses of these mares under as aseptic conditions

as possible. Plate cultures revealed two organisms, the predominating one being the bacillus obtained before from aborting mares in this stud.

Stud No. 2, Case No. 5.—One of the stallions of this stud had a swollen testicle (orchitis) of a chronic type. Pus of this testicle was secured under aseptic conditions and plated, with the result that *B. pseudomonas pyocyaneus* predominated. A few colonies were obtained, however, of the organism associated with infectious abortion in the mares of this stud.

Stud No. 2, Case No. 6.—On December 21, 1911, a male fetus, aborted in the eighth month of gestation, was sent to the laboratory. Cultural and microscopical tests showed the internal organs of this fetus to be invaded with the same bacillus before mentioned.

Stud No. 2, Case No. 7.—On January 8, 1912, a fetus was taken from a mare about to abort in this stud. The results were negative. The afterbirth of this mare was not secured.

Stud No. 2, Case No. 8.—On January 26, 1912, we received a male fetus from a mare aborting in the eighth month of gestation. Streak dilutions, made from the internal organs of this fetus and bits of the afterbirth, gave positive results. A pregnant guinea-pig, inoculated subcutaneously with 4 c.c. of a sterile water suspension of material taken directly from the stomach of the fetus, aborted in 24 hours, and the germ of abortion was isolated from the heart blood and uterus of this pig. The sow had eaten the fetuses, thus giving us no opportunity to determine if the germ could be recovered from them.

Stud No. 3, Case No. 1.—On February 9, 1912, we received a fetus of a mare aborting on this farm in the 226th day of gestation. There was dirt and litter on the afterbirth; therefore a portion of it was first washed with tap water, then with 95 per cent alcohol, and then repeatedly with sterile water. Small pieces of it were then cut up finely and streaked over several dishes of agar, with the result that a large number of colonies were obtained of the germ before mentioned as connected with the afterbirth and fetuses of aborting mares. Platings of the internal organs of the fetus were also positive.

Stud No. 4, Case No. 1.—On December 20, 1912, we examined the exudate from an aborting mare in this stud, with positive results, both microscopically and culturally.

Stud No. 5, Case No. 1.—On December 30, 1912, we examined the exudate from an aborting mare on this farm, with negative results.

Stud No. 4, Case No. 2.—The exudate from an aborting mare was examined, January 6, 1913, with positive results.

Stud No. 5, Case No. 2.—On February 4, the exudate from an aborting mare was examined with positive results.

Stud No. 4, Case No. 3.—The afterbirth and exudate of a mare aborting on February 3, were examined with negative results.

Stud No. 5, Case No. 3.—A mare aborted on February 4. On February 5, the afterbirth and exudate were examined with positive results.

Stud No. 5, Case No. 4.—On February 10 a mare aborted, and an examination of the uterine exudate gave positive results.

Stud No. 5, Case No. 5.—On February 11, the afterbirth of an aborting mare was examined with positive results.

Stud No. 6, Case No. 1.—On February 13, we examined the afterbirth and fetus of an aborting mare with positive results.

Stud No. 5, Case No. 6.—The afterbirth, uterine exudate and fetus of a mare aborting on February 14, 1913, gave positive results.

Stud No. 5, Case No. 7.—The uterine exudate of a mare aborting on February 17 gave positive results.

Stud No. 4, Case No. 4.—The exudate from a mare aborting on February 17 gave positive results.

Stud No. 5, Case No. 8.—The uterine exudate from a mare aborting February 21 gave positive results.

Stud No. 5, Case No. 9.—Uterine exudate of a mare delivering a live foal, but showing a characteristic chocolate colored discharge from the genitals, was examined on March 4 with positive results.

In both seasons, we examined a number of fetuses and afterbirths of mares, supposed to have aborted from accident or multiparous pregnancy, in studs where infectious abortion did not exist. In nearly every instance when examined soon after being aborted, the internal organs of these fetuses were found to be sterile. The bacillus of abortion in mares was not found in any of this material. *B. coli communis* was found associated in large numbers with two small aborted embryos.

In all these investigations, the uterine exudate and material from the fetuses of aborting mares in infected studs have been subjected to the cultural conditions needed for the growth of *B. abortus* Bang, the organism causing the disease in the cow, but in no instance at this laboratory have we found that germ, though we have repeatedly isolated it from the uterine exudates and fetuses of aborting cows.¹ Neither has the streptococcus, mentioned by Ostertag as being associated with infectious abortion in mares, been observed. Many plates streaked with material were incubated under anaerobic conditions, with no further developments.

During the summer of 1912, Dr. Willis Wilson of the state of Washington notified this laboratory that he had found a diplococcus in abundance in the afterbirth of an aborting mare in his section of the country, where the disease of abortion among mares was existing. A culture sent to this laboratory by Dr. Wilson revealed the same organism that had been isolated at this laboratory from aborting mares. It conformed to some strains isolated here, in that the germ had a tendency to stain at the ends, thus making it appear like a diplococcus. This tendency is sometimes noted in

¹ *Bull. No. 165, Ky. Agri. Exper. Sta., 1912, p. 225.*

examining old cultures. The organism corresponded to all the cultural characteristics of the strains isolated at this laboratory. It also behaved identically in agglutination tests.

INOCULATION EXPERIMENTS.

On July 23, 1912, an intravenous injection of a suspension of this organism into the ear vein of a rabbit produced death in 1 hour. Subcutaneous inoculations of contents of a fetal stomach produced



FIG. 4.—*Bacillus abortus equinus*; surface colonies. $\times 4$.

abortion in the guinea-pig in about 36 hours. On Feb. 13, 1913, 4 c.c. of a broth suspension of this organism injected intravenously into each of two hogs produced lassitude, loss of appetite, and a decided rise of temperature. The hogs entirely recovered, however, in three or four days' time.

On March 6, 1912, an intravenous injection of a similar amount of the organism in a pregnant ewe produced abortion in 72 hours. The organism was isolated from all the internal organs of the fetus and from the afterbirth of the ewe.

On February 15, 1913, a subcutaneous injection of 4 c.c. of a physiological salt suspension of the organism in a pregnant ewe did not produce abortion.

On May 10, 1912, an intravenous injection of 20 c.c. of a physiological salt suspension of the organism produced abortion in a pregnant 250-pound sow in 41 hours. The organism was recovered from the afterbirth but not from the fetuses.

On February 7, 1912, a supposedly pregnant mare was slowly injected intravenously with 10 c.c. culture suspension and found afterward not to be in foal. This injection nearly proved fatal to the mare, as the following day her temperature rose to 108 degrees. She soon recovered, however.

On February 28, 1913, 10 different strains of the bacillus isolated from aborting mares were washed off agar slants with physiological salt solution and 2 c.c. injected intravenously into a pregnant mare. The preinjection temperature of this mare was 99.8° F. At the end of five hours it registered 103.8° F. At the end of 21 hours after inoculation her temperature was again normal and continued normal until she aborted. On March 10, this mare aborted without any seeming inconvenience. The afterbirth and internal organs of the fetus were typical of cases where natural infection existed. The organism was found in pure culture and in abundance in the heart, liver, lungs, spleen, kidneys, stomach, lymphatics, intestines, and testicles of this fetus. The germ was also isolated in abundance from the afterbirth and from the contents of the uterus. The history of this mare was known. She was eight years of age, had delivered four normal foals, and had never aborted. One of the writers took care of the mare after treatment, and at no time after the second day of the inoculation did she show any abnormal symptoms or temperature. She gave no indication at any time that she would abort. Soon after the mare aborted the genital tract was flushed with a one per cent solution of creolin, and on three subsequent days with a saturated solution of boric acid. The mare was somewhat depressed for two days after aborting. Her appetite, however, was nearly normal. After the third day, she appeared normal in every way and took the stallion the eighth day after aborting. There was some chocolate colored discharge

from the vagina for six days after slipping. Cultures made from this discharge, taken from the vagina, revealed the abortion bacillus up to the fifth day after aborting. From that time on, all tests were negative, as far as securing a culture of this organism.

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF THE ORGANISM.

Morphology and Staining.—In the tissues, the organism resembles a coccus and a short plump bacillus with rounded ends measuring 0.3 to 0.5μ in width and 0.5 to 1.0μ in length. When



FIG. 5.—*Bacillus abortus equinus*. $\times 1000$.



FIG. 6.—*Bacillus abortus equinus*. $\times 1800$.

cultivated, there is some increase in size. The organism stains somewhat irregularly. Some stained preparations show many bacilli staining at the ends. In routine work at this laboratory, we stain with carbol-fuchsin for from one to two seconds. The organism is gram-negative.

Sporulation.—No spores produced.

Motility.—It is slightly motile when the germ is examined from the drop of condensation of an 18-hour-old agar slant culture.

Petri-dish culture.—Surface colonies 24 hours old are round, measuring from a pin point to one-fourth of one millimetre in diameter. To the naked eye, these colonies are glistening, homogeneous and transparent to direct light, and of a bluish-gray color to reflected light. When incubated for two or three days at $37^{\circ} \text{C}.$

the colonies show a marked contrast to a young culture, as they are dull, granular, and of a grayish color. If the colonies are few in number and well separated, they sometimes attain a size of 2-5 mm. in diameter and may become irregular in outline. The edges of some colonies appear as if they had been pressed flat, other colonies assume crater forms, and still others may be concentrically ringed. Occasionally, a typical rose-shaped colony is noted. Large colonies of some



FIG. 7.—*B. abortus equinus*; broth culture.

strains have a decided tendency to wrinkle. In attempting to remove a portion of one of these colonies with a platinum needle, it is found to be tenacious and bits of the same are obtained with some difficulty. A whole colony will at times slide along on the surface of the medium, if pushed with a needle. If the surface colonies are numerous, they increase but little in size, the borders are regular and

the contents granular and homogeneous. All the foregoing description pertains to colonies obtained by streak dilutions of material direct from the tissues.

Gelatin.—Does not liquefy gelatin; stab culture filiform.

Broth.—After a few days' time, if incubated at 37°C ., a whitish, wrinkled pellicle forms at the surface, which, upon shaking slightly, settles to the bottom of the tube en masse.

Milk.—Does not coagulate milk even on being incubated 10 days, and the end of reaction is alkaline to litmus.

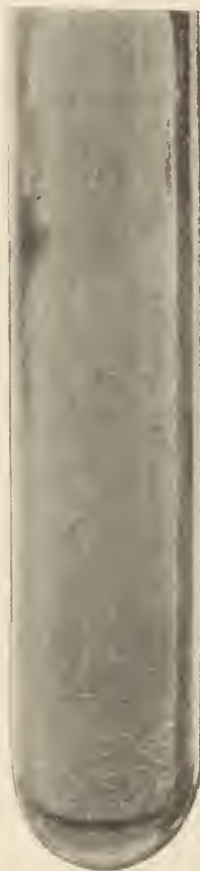


FIG. 8.—*B. abortus equinus*. Streak on plain agar. Actual size.

Agar slants.—Streaks of the bacillus on agar slants are very characteristic, in that after the tubes are incubated for a day or two the growth becomes dull and parchment like, and usually develops thin wrinkles near the drop of condensation. The drop of condensation is covered with a film of growth.

Physiology.—The organism is aerobic and facultative anaerobic. Dextrose, sorbit, dulcit, mannite, xylose, arabinose, and raffinose are fermented with the production of both acid and gas. While we have reported that this organism does not ferment lactose and saccharose, we have since determined that most of our strains now produce a trace of acid and gas in broth containing one per cent of these sugars. Adonit, sorbose, and rhamnose are not fermented. Our investigations, so far, tend to show that while sorbose yields no gas, a slight amount of acid is produced. All broth to which sugars had been added was made from an infusion of beef. The beef infusion was rendered free from muscle sugar according to the usual technic, by inoculation with *B. coli communis*. The germ produces six per cent of gas in Jackson's bile tube in 48 hours.

Indol was not produced.

Although these fermentation tests were somewhat extensive, we wish to state that we do not deem the results tabulated as exactly typical of all strains of this organism. More work is now being done along this line.

AGGLUTINATION AND COMPLEMENT FIXATION TESTS.¹

Tables 1 and 2 give the results of agglutination and complement fixation tests of blood from mares which had aborted, as well as blood from non-aborting mares in non-infected studs.

On March 10, 1913, blood was drawn from 13 of 28 aborting mares (Nos. 1 to 13 in Stud No. 5, Tables 1 and 2). Darkness prevented blood being taken from the entire 28 at that time. These mares aborted at different intervals between December 1, 1912, and February 25, 1913. Numbers 19 and 19a represent the test of blood of the mare before and after abortion was produced experimentally. The second blood drawing was taken 8 days after aborting.

¹ Dr. F. M. Surface of this station kindly ran the complement fixation test for the writers.

As can be seen from Table 1, the blood serum of aborting mares (Nos. 1 to 13) agglutinated the bacillus in dilutions varying from

TABLE 1.*

SHOWING AGGLUTINATION TESTS WITH THE BACILLUS FOUND CONNECTED WITH INFECTIOUS ABORTION IN MARES.

Final Reading After 60 Hours' Incubation.

Source of Blood Serum. Number	1-200	1-300	1-500	1-800	1-1000	1-1500	1-2000	1-3000	Check
1. Aborting Mare.....	+	+	+	-	o	o	o	o	o
2. " ".....	+	+	+	+	+	+	+	+	o
3. " ".....	+	+	+	+	+	+	+	+	o
4. " ".....	+	+	+	+	+	+	+	+	o
5. " ".....	+	+	+	+	+	+	+	+	o
6. " ".....	+	+	+	+	+	+	+	+	o
7. " ".....	+	+	+	+	+	+	+	+	o
8. " ".....	+	+	+	+	-	o	o	o	o
9. " ".....	+	+	+	+	+	+	-	-	o
10. " ".....	+	+	+	+	+	+	+	+	o
11. " ".....	+	+	+	+	+	+	+	+	o
12. " ".....	+	+	+	+	+	-	o	o	o
13. " ".....	+	+	+	+	+	o	o	o	o
14. Normal.....	+	-	o	o	o	o	o	o	o
15. " ".....	+	-	o	o	o	o	o	o	o
16. " ".....	+	o	o	o	o	o	o	o	o
17. " ".....	+	o	o	o	o	o	o	o	o
18. " ".....	+	o	o	o	o	o	o	o	o
19. Before aborting.....	+	o	o	o	o	o	o	o	o
19a. After aborting.....	Gave plus agglutination 1:5000. minus at 1:8000.								

* In the table the sign (+) indicates a complete agglutination; the sign (-) indicates that the reaction has progressed considerably, but not a clearing up of the turbidity of the antigen; and the sign (o) indicates no change.

TABLE 2.*

COMPLEMENT FIXATION TESTS.

NUMBER OF HORSE	AMOUNT OF INACTIVATED HORSE SERUM				
	0.2 C.C. Control without Antigen	0.1 C.C.	0.05 C.C.	0.03 C.C.	0.01 C.C.
1.....	++++	o	++	++++	++++
2.....	++++	o	o	o	o
3.....	++++	o	o	o	++
4.....	++++	o	o	o	o
5.....	++++	o	o	o	o
6.....	++++	o	o	o	o
7.....	++++	o	o	o	o
8.....	++++	o	o	o	o
9.....	++++	o	o	o	o
10.....	++++	o	o	o	o
11.....	++++	+++	+	+	+
12.....	++++	o	o	o	o
13.....	++++	o	o	o	o
14.....	++++	++++	++++	+++	+++
15.....	++++	++++	++++	++	++
16.....	++++	++++	++++	++++	++++
17.....	++++	++++	++++	++++	++++
18.....	++++	++++	++++	++++	++++
19.....	++++	++++	++++	+++	++
19a.....	++++	++	o	o	o

* In this table, ++++ means complete hemolysis; +++ nearly complete solution; ++ partial hemolysis; + a trace of hemolysis; while o indicates complete fixation of the complement.

1:500 in No. 1, to 1:3,000 in Nos. 2, 3, and 11. No. 19a, in which abortion was produced experimentally, gave complete agglutination 1:5,000 and considerable reaction in a dilution of 1:8,000. The blood from non-infected horses (Nos. 14 to 18, inclusive, Tables 1 and 2) agglutinates 1:200 with considerable reaction, in two instances at 1:300.

Dr. Surface comments on Table 2 as follows:

"1. Each of the first 13 mares shows some fixation of the complement. Mares No. 1 and 11 do not show as marked fixation as the others.

"2. Mares No. 14, 15, and 16 came from a farm where there had been no abortion. Nos. 14 and 15 show some tendency to inhibit the hemolysis, but these could not be regarded as definite fixations. Likewise, Nos. 17, 18, and 19, horses which had never been exposed to the disease, show no reaction.

"3. Thus, all the 13 mares which aborted respond positively to the fixation test, while the five mares from non-infected farms react negatively.

"4. Mare No. 19, when tested before inoculation, showed no definite fixation, although small amounts of serum appeared to inhibit the hemolysis to a slight extent. Seventeen days after inoculation (No. 19a), she showed marked fixation of the complement."

As this paper was being written, Dr. Surface called the attention of the writers to an article¹ by Professor Dr. D. A. de Jong of Leiden, Holland. In October, 1911, his attention was called to an outbreak of infectious abortion in one of the provinces of his country. He stated that the abortions came without alarming previous symptoms and with no injurious sequelae, the mares being able to work soon after aborting. Dr. de Jong secured a culture of a short, oval bacillus in abundance from the internal organs of two aborted fetuses, as well as from the fetal membranes. He found the bacillus in pure culture.

Dr. de Jong placed the bacillus in the *Paratyphus B. enteritidis* group, from the following characteristics: (1) bacillus motile; (2) gelatin not liquefied; (3) milk not coagulated; (4) grape sugar fermented; (5) saccharose fermented; (6) milk sugar not fermented; (7) litmus milk turned first red, then blue; (8) decolorized neutral red with florescence; (9) no indol formed. He called attention to the tendency of a streak on agar to wrinkle near the drop of condensation.

Dr. de Jong was also able to produce abortion experimentally in mares, by intravenous injection of a culture suspension, the incubation period being 11 days in one instance, and 30 days in another instance. The organism was recovered from the fetus and afterbirth in each instance. He was able, also, to produce the disease by feeding, the incubation period being 13 days, and the organism was recovered from the fetus and afterbirth.

SUMMARY.

1. Infectious epizootic abortion is noted from time to time among the mares of this region as well as in other parts of the United States.

¹ *Centralbl. f. Bakteriöl.* 1912, I Orig., 67, p. 148.

2. The etiology of the disease of infectious abortion in the mare, until recently, has been indefinite.

3. From the afterbirths, fetuses and uterine exudates in five studs of aborting mares and one stud of aborting jennets, this laboratory has isolated a germ belonging to Subgroup II of the Colon typhoid group, to which subgroup belong *B. cholera suis* and *B. enteritidis*.

4. In our investigations, *B. abortus* Bang has not been found associated with the disease of infectious abortion in the mare, although we have often isolated the same from aborting cows. Neither has the streptococcus, observed by Ostertag, been found.

5. While we place the bacillus isolated at this laboratory in the same group as *B. enteritidis* and *B. cholera suis*, it differs somewhat in cultural characteristics, and serum immune to the bacillus obtained from the mare does not agglutinate either *B. enteritidis* or *B. cholera suis*.

6. As it is evident that the etiological factors of the disease of infectious abortion of the cow and of the mare are different, we would suggest that the bacillus causing abortion in the mare be differentiated from *B. abortus* Bang by naming it *B. abortivus equinus*.

7. From our investigations, normal horse serum agglutinates *B. abortivus equinus* in a dilution of 1:200, occasionally 1:300, while the serum of infected animals agglutinates from 1:500 to 1:5,000.

8. The results of the work of Dr. F. M. Surface show that the serum of infected animals fixes the complement.

9. A ewe and a sow, injected intravenously, and guinea-pigs, subcutaneously, with pure cultures of this germ, aborted and the organism was recovered from the uterine exudates.

10. An injection of two cubic centimeters of physiological salt suspension of *B. abortivus equinus* in a pregnant mare caused abortion in 10 days, with typical symptoms of natural infection. The organism was recovered in abundance from all the internal organs of the fetus, as well as the fetal membranes and uterus of the mare.

SERUM STUDIES IN PNEUMONIA.

THE ANTIGENIC PROPERTIES OF FIBRIN (EXUDATE) TO SERUM.

C. C. HARTMAN.

The R. B. Mellon Fellow in Pathology.

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The object of the work here reported was to determine, by the means at hand, the presence of antibodies in the serum of patients with pneumonia, which might assist in the removal of the fibrinous exudate during resolution. We started out on the supposition that human fibrin is more or less identical, though the possibility of its variation in diseased conditions was kept in mind.

Lamar and Meltzer¹ have reported the successful production of lobar pneumonia in dogs by intratracheal insufflation; Wollstein and Meltzer,² that of bronchopneumonia, noting the difference in the fibrin content of the exudate, distinguishing true lobar pneumonia from others, as well as appreciating the difference in the distribution of the inflammation, the appearance of the reaction, the resolution, and the mortality. The pneumococcus seems to call forth a reaction in the lung sufficiently characteristic to allow of its differentiation from other organisms. Lamar³ has shown the modifying effect of the soaps on pneumococci, indicating their greater susceptibility to autolysis and to serum lysis after being acted upon by the sodium oleate. Rosenow⁴ has observed the toxic qualities of pneumococci autolysates and their power to call forth antibodies. Dick⁵ has shown the development of proteoclastic ferments specific for extracts of pneumococci in the blood during pneumonia. Barker⁶ has shown the presence of proteoclastic enzymes in fibrinous exudates and fibrin. Opie⁷ particularly has observed the proteoclastic enzymes in leukocytes, and many others have worked with different phases of immunity concerned in pneumonia.

Since fibrin is such a constant and characteristic component of the exudate in pneumococcus lobar pneumonia and distinguishes

* Received for publication April 29, 1913.

¹ *Jour. Exper. Med.*, 1912, 15, p. 133.

² *Ibid.*, 1912, 16, p. 126.

³ *Ibid.*, 1911, 13, p. 1.

⁴ *Jour. Infect. Dis.*, 1911, 9, p. 190.

⁵ *Ibid.*, 1912, 10, p. 282.

⁶ *Jour. Exper. Med.*, 1908, 10, p. 343.

⁷ *Ibid.*, 1906, 8, p. 410.

it more or less from lung exudates caused by other organisms, as well as from exudates elsewhere in the body caused by the same organism (pneumococcus), the possibility of its playing more than a mere passive part in the process occurred to us worthy of investigation; especially, since, in pneumococcus infections elsewhere, fibrin does not appear in such an abundance and in them the course is anything but analogous to that which occurs when the lung is the seat of inflammation. The situation of the infection scarcely accounts for the difference, because other infections in the lung differ in character, distribution, and course, as shown by Lamar and Meltzer, and Wollstein and Meltzer, and as has been known pathologically for a long time.

Aside from the enzymes associated with it, fibrin has been considered to be more or less an inert material, being constant in composition regardless of the location or the condition under which it arises. Its predominance in the exudate of lobar pneumonia, its purpose in the reaction, the method of its disappearance, must be explained in order fully to understand those interesting phenomena of crisis and resolution. One of the striking points in the histological picture of a consolidated lung after crisis is the beginning of melting away or digestion of the strands of fibrin just within the alveolar wall.¹ Since leukocytes contain proteoclastic enzymes, as do fibrinous exudates and blood serum, the disappearance of the fibrin would be readily explained along these lines, but it has not been demonstrated that this is actually the manner in which it disappears. Phagocytosis is considered to play little part in the disappearance of either the fibrin or pneumococci.² The beginning of fibrinolysis at the periphery of the alveolar plug and its appearance immediately after the crisis suggested that something might be furnished by the blood serum which aids in the destruction of the fibrin, and that this might be shown *in vitro*.

METHODS.

The fibrin was obtained by whipping specimens of blood taken from superficial veins in the arm by the use of a syringe. The fibrin mass was washed in sterile, distilled water until the hemo-

¹ Ziegler, *Pathological Anatomy*, London, 1898, p. 833.

² Rosenow, *Jour. Infect. Dis.*, 1911, 8, p. 500.

globin ceased to come out. It was then placed on watch crystals, the excess water being allowed to drain off, and kept in a desiccator over potash at room temperature. One specimen, however, that from Case 7, was recovered from a clot. The fibrin, as needed, was ground to a fine powder in a mortar and suspended in a quantity of salt solution, equal to the amount of blood from which it was derived. Usually there remained enough hemoglobin attached to the fibrin to give the powder a tinge of brown. Everything was carried out with a constant view to asepsis.

The experiments were based on the Bordet-Gengou phenomenon of complement fixation and patterned after the Noguchi modification of the Wassermann reaction. The fibrin suspension was used as antigen. The complement was derived from fresh guinea-pig serum and used in an amount necessary to cause complete hemolysis of 1 c.c. of a 1 per cent suspension of human cells in the presence of a standard unit of amboceptor in one hour. The red cells were, with but one or two exceptions, from a constant source.

The fibrin suspension, serum to be tested, and complement were measured out by separate, sterile, capillary pipettes and were added in the order given. All tubes were incubated either one or two hours before the addition of the amboceptor at 37° C., and were shaken thoroughly every half-hour. The final reading was taken at the end of two hours after the addition of the amboceptor.

It might be well to state that there seemed to be a greater amount of fibrin in the pneumonic blood than in corresponding quantities of normal blood (surgical cases). This impression, gleaned only from casual observation in the gross and not controlled by actual weights, is offered merely as an impression. Whether this appearance was due to a difference in the compactness of the fibrin shreds or to some other factor, we do not know. However, in view of the fact that fibrinogen is present in the serum in pneumonia in increased quantities,¹ this observation may be borne out by subsequent work.

The serum was obtained either by centrifuging the whipped blood from which the fibrin had been taken, or pipetted from blood allowed to clot. It was kept either in sterile test-tubes or sterile

¹ Dochez, *Jour. Exper. Med.*, 1912, 16, p. 665.

pipettes in a refrigerator. Thirty specimens of serum and one of pericardial fluid from pneumonia patients were used, one to four specimens being obtained from each patient. It was the intention to get specimens of serum at various periods of the disease and to try them against various specimens of fibrin. As controls, 20 specimens of serum from convalescing surgical patients were used.

ANALYSIS OF CASES.

In this series of cases, there were 21 adults, 20 males and 1 female, ranging in age from 17 to 55 years. Fifteen recovered and six died. A few of these cases were complicated by other conditions.

TABLE 1.
FIBRIN FROM NON-PNEUMONIA PATIENTS.

No.	Fibrin	Serum	Day of Disease	Termination	Lysis or Crisis	Result
1.....	A	Case 4	7th	Recovered	Lysis	No binding
2.....	A	Case 4	10th	"	"	" "
3.....	A	Case 4	12th	"	"	" "
4.....	A	Case 5	11th	Died	Slight binding
5.....	A	Normal (A)	No binding
6.....	A	Normal (B)	" "
7.....	A	Case 5	11th	Died	" "
8.....	A	Case 5*	15th	"	Complete binding
9.....	B	Case 7	5th	"	No binding
10.....	B	Case 6	11th	Recovered	Lysis	" "
11.....	B	Normal (R)	" "
12.....	B	Case 4	12th	"	Lysis	" "
13.....	B	Case 4	10th	"	"	" "
14.....	B	Normal (A)	" "
15.....	B	Normal (B)	" "
16.....	C	Case 7	5th	Died	" "
17.....	C	Case 8 *	9th	"	" "
18.....	C	Case 6	11th	Recovered	Lysis	" "
19.....	C	Normal (M)	" "
20.....	C	Normal (B)	" "
21.....	D	Case 9	?	Died	" "
22.....	D	Case 11	11th	Recovered	Lysis	Slight binding
23.....	D	Case 12	9th	"	"	No binding
24.....	D	Case 13	15th	"	"	" "
25.....	D	Case 7	5th	Died	" "
26.....	D	Normal (A)	" "
27.....	D	Normal (B)	" "
28.....	E	Case 14	10th	Recovered	Lysis	Slight binding
29.....	E	Case 15	2d	"	"	No binding
30.....	E	Normal (C)	" "

* Pericardial fluid.

Case 9 developed a terminal pneumonia on an apical tuberculosis and chronic heart disease; Case 2 was suspected of having pulmonary tuberculosis, and had a tuberculous process of the vertebrae with abscess formation. Cases 2, 4, 6, 19, 21, and 22 had a history of alcoholism. There was no definite causal relationship

between the alcoholism and the pneumonia in Cases 6, 21, and 22, so that it is possible to consider 18 of the cases as true lobar pneumonia. Among the 21 cases, crisis occurred but twice (16 and 23).

The five specimens of fibrin were derived from the blood of the following patients: A, having an abscess of the thigh; B, a simple fracture; C, a minor injury; D, recovering from a herniotomy; E, having an unimportant condition.

The table represents a total of 42 trials of the five specimens of fibrin against the sera of 19 individuals, and a pericardial fluid. Thirteen of the sera and the pericardial fluid were from patients

TABLE 2.
FIBRIN FROM PNEUMONIA PATIENTS.

No.	Fibrin	Serum	Day of Disease	Termination	Lysis or Crisis	Result
1	Case 1	Case 1	7th	Died	No binding
2	Case 1	Case 2	13th	Recovered	Lysis	" "
3	Case 1	Case 2	15th	"	"	" "
4	Case 1	Normal	Abscess of Thigh	"	" "
5	Case 1	Case 4	7th	Recovered	Lysis	" "
6	Case 1	Normal (Ben)	"	" "
7	Case 1	Normal (O W)	"	" "
8	Case 1	Normal (Shupe)	"	Slight binding
9	Case 1	Normal (Fred)	"	No binding
10	Case 1	Normal (Mur)	"	" "
11	Case 2	Case 4	7th	Recovered	Lysis	" "
12	Case 2	Case 4	10th	"	"	" "
13	Case 2	Normal (Shupe)	"	Slight binding
14	Case 2	Normal (Fred)	"	No binding
15	Case 2	Normal (Mur)	"	" "
16	Case 2	Case 5	11th	"	Complete binding
17	Case 4	Case 6	11th	Recovered	Lysis	" "
18	Case 4	Case 5*	15th	Died	"	" "
19	Case 4	Case 4	12th	Recovered	Lysis	" "
20	Case 4	Case 4	10th	"	"	" "
21	Case 4	Normal (A)	"	" "
22	Case 4	Normal (Own)	"	Partial binding
23	Case 4	Normal (B)	"	Complete binding
24	Case 7	Case 7	5th	Died	"	No binding
25	Case 7	Case 8	9th	"	"	" "
26	Case 7	Case 6	11th	Recovered	Lysis	" "
27	Case 7	Normal (M)	"	" "
28	Case 7	Case 7	5th	Died	"	" "
29	Case 7	Normal (A)	"	" "

* Pericardial Fluid.

having pneumonia, and 6 from control patients. Binding of the complement was observed 4 times, with the serum (11th day) and pericardial fluid (15th day) of Case 5, with the serum of Case 11 (11th day) and with that of Case 14 (15th day). The binding of the pericardial fluid of Case 5 was complete; that of the 3 sera

slight. The sera from the cases of pneumonia varied in relation to the period of the disease from the second to the fifteenth day. The temperature fell by lysis in all cases recovering; 4 patients died. The binding of complement by the pericardial fluid may be analogous to the positive Wassermann reaction obtained in the fluid of the cadaver.

The pneumonia fibrin used in these experiments was obtained from the following; Case 1, 7th day; Case 2, 13th day; Case 4, 7th day; Case 7, 5th day.

This table represents the results of 34 trials of the different specimens of fibrin against 20 sera and one pericardial fluid, 10 sera and the pericardial fluid being from cases of pneumonia and 10 sera used as controls. The pneumonia sera varied in relation to the period of the disease from the fifth to the fifteenth day. Three patients died. In those recovering the temperature fell by lysis in all.

Binding of complement occurred 10 times, 5 times by control sera and 5 by pneumonia sera. In 7 instances, binding occurred in the presence of the fibrin of Case 4.

In order to simplify technic it was decided to powder a number of specimens of fibrin, mix and suspend them in alcohol and ether (9-1) to serve as a stock supply from which amounts necessary for each day's work could be measured out. The stock suspension was made so that 1 c.c. contained an amount of fibrin equal to that derived from 5 c.c. of blood, and when diluted with 4 parts saline equaled the strength generally used in the experiments. It contained the fibrin derived from the specimens of blood from the following cases: Case 6, 11th day; Case 8, 9th day; Case 11, 11th day; Case 12, 8th day; Case 14, 9th day; Case 15, 2d day.

This mixed fibrin was used as antigen against 19 sera, 14 from pneumonia and 5 from control patients. In the 13 cases which recovered, the temperature fell by lysis in 10, by crisis in 2, and by protracted crisis in 1. The specimens of sera covered a wide range in the period of the disease—from the sixth to the twenty-sixth day. The controls included serum from a patient with known syphilis and a positive Wassermann. There was no binding whatever observed in this series.

That fatty substances are present in inflammatory processes is well known, and that lipase is present has also been indicated by Flexner.¹ The modifying effect of fatty

¹ *Jour. State Med.*, 1912, 20, p. 193.

substances upon bacteria, shown by Lamar, suggested the possibility of their having a similar effect upon fibrin. An attempt to demonstrate this was made by subjecting specimens of fibrin to the action of sodium oleate (Merck) in 1-1,000 solution over night at room temperature. The fibrin was thrown down by the centrifuge, washed once in saline, and resuspended in saline to the approximate strength used in the previous experiments.

TABLE 3.
MIXED FIBRIN—KEPT IN ALCOHOL AND ETHER.

No.	Serum	Day of Disease	Termination	Lysis or Crisis	Result
1.....	Case 16	7th	Recovered	Crisis 8th day	No binding
2.....	Normal (D)	" "
3.....	Case 16	14th	Recovered	Crisis 8th day	" "
4.....	Normal (E)	" "
5.....	Case 16	18th	Recovered	Crisis 8th day	" "
6.....	Case 17	7th	"	Lysis	" "
7.....	Case 18	7th	"	"	" "
8.....	Case 16	26th	"	Crisis 8th day	" "
9.....	Case 17	15th	"	Lysis	" "
10.....	Case 18	14th	"	"	" "
11.....	Case 19	11th	"	"	" "
12.....	Normal (F)	" "
13.....	Case 19	24th	Recovered	Lysis	" "
14.....	Case 20	7th	"	Protracted crisis	" "
15.....	Case 21	7th	"	Lysis	" "
16.....	Case 22	6th	Died	" "
17.....	Normal (G)	" "
18.....	Normal (Sy- philitic (H)	" "
19.....	Case 18	4th day after crisis	Recovered	Crisis? day	" "

TABLE 4.
SOAPED FIBRIN.

No.	Serum	Day of Disease	Termination	Lysis or Crisis	Result
1.....	Case 16	7th	Recovered	Crisis 8th day	No binding
2.....	Normal (D)	" "
3.....	Case 16	7th	Recovered	Crisis 8th day	" "
4.....	Case 16	14th	"	" " "	" "
5.....	Case 16	18th	"	"	" "
6.....	Case 17	6-7th	"	Lysis	" "
7.....	Case 18	7th	"	"	Slight binding
8.....	Normal (I)	No binding

The fibrin, subjected to the action of sodium oleate, was tried against 7 different sera—5 pneumonia and 2 control. All of the pneumonia patients recovered, one having a crisis. A slight binding by the serum of Case 18 was the only instance of binding observed.

Experiments were also undertaken to determine the presence of antibodies in the serum to the decomposition products of fibrin.

A weighed amount of powdered fibrin (0.05 gm.), approximately equal to the average amount derived from 5 c.c. of blood, was mixed with a suspension of fresh pus cells from an empyema in distilled water, a little chloroform added, and the whole placed in an incubator at 37° C. for 60 to 70 hours. A similar amount of fibrin was incubated for 20 hours, with pus cells from the same source, but having been preserved

in 50 per cent glycerin for 2 days. After incubating, the remains of fibrin and pus cells were thrown down by the centrifuge and the supernatant liquid pipetted off. This was used as antigen in amounts of 3 to 5 drops.

TABLE 5.
PRODUCTS OF FIBRIN DIGESTION.

No.	Serum	Day of Disease	Termination	Lysis or Crisis	Result
1.....	Case 10	24th	Recovered	Lysis	No binding
2.....	Case 20	7th	"	Protracted crisis	" "
3.....	Case 21	7th	"	Lysis	" "
4.....	Case 22	6th	Died	"	" "
5.....	Normal (G)	" "
6.....	Normal (H) (syphilitic)	" "

The products of digestion of fibrin by leukocytes were used against 6 sera—4 pneumonia and 2 control. The pneumonia sera varied in relation to the stage of the disease from the sixth to the twenty-fourth day. Binding did not occur in any instance.

If we eliminate from our various experiments all those reactions which were at all questionable, that is, where binding was not marked, there remain several definite and distinct observations which seem to be worthy of consideration.

The fibrin of Case 4, obtained about the seventh day of the disease, bound complement more or less completely with every serum, pneumonia and otherwise, against which it was tried. Some of these sera had shown no binding with several other specimens of fibrin. This makes it appear that this specimen of fibrin differed in some respects from the other fibrins against which these sera had been tried. The stage of the disease at which the fibrin was obtained was similar to that of Case 1, which did not show this reaction with any sera. Case 1 was a female who died in her illness, while Case 4 was a male who recovered. The fibrin from Case 1 was kept 11 days before using and that of Case 4, 12 days. Just wherein the difference of reaction lies, we are not prepared to say. Whether it is a substance mechanically adhering to the fibrin, or forming a combination with it, or a change in the composition or structure of the fibrin itself, is another problem. In connection with some other experiments in progress, the possibility of this binding action being due to associated fatty substances suggested itself.

The serum of Case 5 partially bound the complement in the

presence of one non-pneumonia fibrin and completely in the presence of one pneumonia fibrin. The pericardial fluid taken from the same case at autopsy was positive in the presence of a non-pneumonia fibrin and the fibrin of Case 4.

The serum of pneumonia patients did not indicate a greater ability as shown in the experiments, before or after crisis or lysis, to bind complement in the presence of fibrin. In fact, the serum showing the greatest ability to bind complement in the presence of different fibrins was from a fatal case (No. 5), about the eleventh and fifteenth days of the disease before the temperature had fallen. The antigenic property of fibrin appears to bear no relation to the state of the disease when collected, nor does it vary with the subsequent course of the disease. Thus too, it was found that the serum of Case 5 reacted with both the fibrin of a control patient and that from a case of pneumonia.

Two attempts were made to immunize rabbits against fibrin. In the first, two rabbits were injected intraperitoneally with successive injections of powdered fibrin held in suspension in saline. After three injections, given at interval of four days, of 0.118 gms. fibrin each (approximately equal to that from 10 c.c. of blood), both of the rabbits died. A toxic peritonitis was the main finding at autopsy. All cultures were negative.

Another attempt was made with two rabbits, using smaller amounts of fibrin. The dry powdered fibrin was sterilized at 56° C. It was injected intraperitoneally in amounts varying from 0.0125 gm. to 0.025 gm., every second or third day for nine doses. Serum taken at various times did not show any antibodies toward fibrin. Neither were any fibrinolytic substances demonstrated in the serum. The serum did not possess any fibrinolytic substances when tested against pure fibrin or suspension of fibrin in agar (in petri plates).

We have found little assistance in the literature bearing directly upon our problem, though in some work done by Müller (quoted by Kossel)¹ some interesting points were brought out, indicating peptone in pneumonic sputum after crisis, but never before. He also observed the presence of a ferment in all pus-containing sputum capable of digesting fibrin, and that serous sputa and pneumonic sputa, before crisis, did not possess this power.

¹ *Ztschr. f. klin. Med.*, 1888, 13, p. 149.

The question of the formation of auto-antibodies is rather unsettled, though not a few believe that they may occur and have been satisfactorily demonstrated. The great ability of the serum from Case 5 to react with fibrin and bind complement, as shown in our experiments, suggests that some of the toxic effects of the disease might be due to products of fibrin digestion with the appearance of antibodies in the circulation. Such decomposition products of fibrin have been shown to be toxic for animals.

GENERAL DISCUSSION.

Fibrin, in some instances, appears to be able to bind complement in the presence of serum, but the results are so irregular that they cannot be considered to be specific. Nevertheless, it is the indication of a process, the object of which is the digestion or lysis of the fibrin. Looking at it from the standpoint of auto-antibody formation, it is possible to view it as a reaction on the part of the body to rid itself of one of the products of its own manufacture. As such, fibrin does not exist in the body normally and may perhaps, on this account, act as foreign material. When the antibodies arrive in sufficient strength, the fibrinolysis begins, by the aid of complement furnished by the blood serum or leukocytes. Though the body might exhibit marked power to destroy or decompose the fibrinous exudate, in some instances, it may not possess the same ability to deal with the decomposition products.

The fact that a certain number of the "normal" sera reacted with certain specimens of fibrin is of interest, indicating the presence of substances in the serum similar to that found in a few pneumonia sera. It is possible that these reactions are not specific, but are the evidence of a previous inflammatory process of a fibrinous character.

The fact that the remains in non-resolution are principally fibrinous rather than cellular¹ indicates an absence in the body of the means of removing this portion of the exudate. If the disappearance of the fibrin be entirely due to the enzyme action of the leukocytes and of the exudated serum, it seems strange that autolysis or digestion of the exudate, except the fibrin, should go on to

¹ Flexner, *Univ. of Penn. Med. Bull.*, 1903, 16, p. 1.

completion or near completion, in some cases of non-resolution, leaving the fibrin intact. It rather indicates the participation of something else in addition to the autolysis or digestion by leukocytic enzymes. Whether this persistence of the fibrin is due to a lack of preparation or "sensitization" to lysis similar to the opsonic reaction, or to an inherent antienzymotic action, or a lack of something in the body fluids designed especially to remove the fibrin, is not apparent. Though it would appear that a common process brings about the removal of the entire exudate, nevertheless, a persistence of the fibrin in spite of the disappearance of much or all of the associated exudate in some instances, points toward a process differing from that of a leukocytic digestion, though perhaps working in association with it.

The inability to immunize rabbits against fibrin is interesting as indicating that the tissues of this animal do not react to whole fibrin. A greater number of attempts with various technics would be necessary, however, to demonstrate this more fully. It was shown by the methods used that there was no ability conferred upon the serum to decompose the fibrin.

CONCLUSIONS.

1. Human serum sometimes possesses antibodies for fibrin.
2. Fibrin is a variable factor as antigen, some specimens being able to react with many sera and some with few or none.
3. Fibrin kept in alcohol and ether loses its power as antigen.
4. The "soaping" of fibrin does not increase its efficiency as antigen.
5. Products of fibrin digestion by leukocytes do not serve as antigen.
6. Serum before and after crisis or lysis does not differ strikingly in the ability to bind complement in the presence of fibrin.
7. No relation of the ability of fibrin to act as antigen to the stage of the disease in pneumonia was apparent.

I wish to express my indebtedness to Dr. Oskar Klotz for his valuable advice and supervision of the work, and my appreciation to Dr. McMeans of the Mercy Hospital and to the St. Francis Hospital, for aid in obtaining materials.

A NEW PRECISION SYRINGE FOR THE ACCURATE INJECTION OF SMALL QUANTITIES; A HOLDER FOR SUCH SYRINGES AND FOR OTHER PURPOSES.*

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Stanford University, California.)

The syringe here shown consists of a piece of 1 c.c. pipette, one end of which is ground to fit a Luer needle; to the other end is attached an ordinary atomizer bulb from which the outlet valve has been removed.

To fill the syringe, the air is forced out of the bulb as shown in Fig. 2, the inlet valve is closed with thumb or finger (Fig. 3), and the needle is held in the fluid to be taken up. If the hub of the needle is kept above the level of the fluid, any imperfection will be shown by the entrance of air bubbles. If the connection is perfect the fluid rises slowly and steadily. The rising column may be stopped instantly at any point by removing the thumb from the inlet valve and allowing the bulb to fill with air from that direction.



FIG. 1.

When filled, the syringe may be held horizontally without danger of the contents escaping. If the end of the needle is dipped in vaseline the instrument may be placed in any position, even upside down, without change in the level of the fluid. Fig. 4 shows a number of syringes filled and ready for use, one of them upside down.

When an injection is to be made and the needle has been introduced, a slight quick pressure on the valve closes the inlet valve and the fluid is forced out, slowly or rapidly, by steady pressure on the bulb. The amount injected is read off on the scale, and may be determined with great accuracy. The column of fluid in the syringe may be started and stopped any number of times in the course of an injection and is so readily controlled that,

* Received for publication May 27, 1913.

with a little practice, it is very easy to start and stop it two or three times within the $1/100$ division marks on an ordinary 1 c.c. pipette.

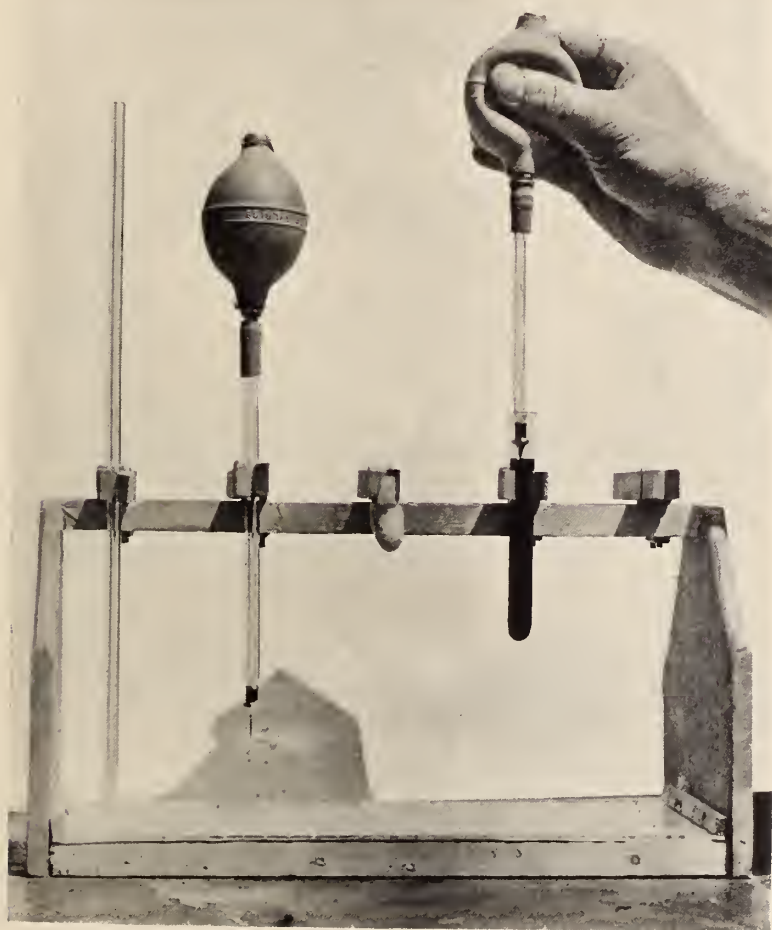


FIG. 2.

The instrument was devised to meet the requirements of an examination into Römer's method of standardizing diphtheria antitoxin in which accurate measurement of the quantities injected

is exceptionally important on account of the high dilutions, the small quantities involved, and the necessity of avoiding unequal pressure effects in the injected areas. A paper on this subject is



FIG. 3.

in preparation, but a brief description of the method and some of the results obtained will be given here to show the suitability of the instrument for purposes requiring exact measurements.

In Römer's method, toxin and antitoxin dilutions are made in

such a manner that the desired quantity of each is contained in 0.05 c.c. of the dilution. Equal parts of the two are mixed and, after a time allowed for combination, are injected *into*, not under, the skin of a guinea-pig. In this way a determination is made of the amount of a given toxin which, in combination with a fixed quantity of a standard antitoxin, produces a minimum degree of necrosis in the area injected. This is called the *Limes Necrosis* dose, and sera to be tested are then titrated against this amount of toxin.

In Table 1 the standard antitoxin was so diluted that the quantity used (0.05 c.c.) contained $1/50$ of an antitoxin unit. Several tests, as shown, are made on each animal.

TABLE 1.

1/50 Antitoxin Unit, Plus Toxin as Below, c.c.	Guinea-Pig No. 13	Guinea-Pig No. 15	Guinea-Pig No. 17
0.005	No necrosis		
0.0055		No necrosis	
0.006		No necrosis	
0.0066		No necrosis	
0.00683			No necrosis.
0.007			Necrosis? Too slight to be sure of.
0.00725			Necrosis, pinhead size, the Limes necrosis.
0.0075	Necrosis, .25 inch in diam- eter		
0.01	Necrosis, more than above		
0.0125	Necrosis, proportionally more than above		

When toxin alone is titrated to determine the minimum intra-cutaneous necrotic dose, still smaller fractions are involved. The importance of accuracy in the measurement of the quantities injected may be judged from Table 2.

TABLE 2.

AMOUNT INJECTED	GUINEA-PIG No. 2		
	After 48 Hrs.	After 4 Days.	After 6 Days.
0.1 c.c. containing 0.0001 c.c. toxin (left front) . . .	Slight necrosis	Necrotic area, $\frac{1}{2}$ in. in diam- eter	No change
0.1 c.c., 0.00075 c.c. toxin (left back)	Infiltration	Necrosis about equal to above	No change
0.0005 c.c. toxin (right front) — 0.1 c.c.	Infiltration	Distinct necrosis less than $\frac{1}{2}$ inch	No change—the min. ne- crotic dose
0.1 c.c., 0.00025 c.c. toxin (right back)	Infiltration	Tissuepaper-thin scaling of skin	No change

Römer used, as did Lewin who reviewed Römer's work, a 1 c.c. Pravatz syringe graduated in 20ths. Similar syringes in this laboratory did not seem adequate to the technic; nor could anything better be found in San Francisco. The one here described has

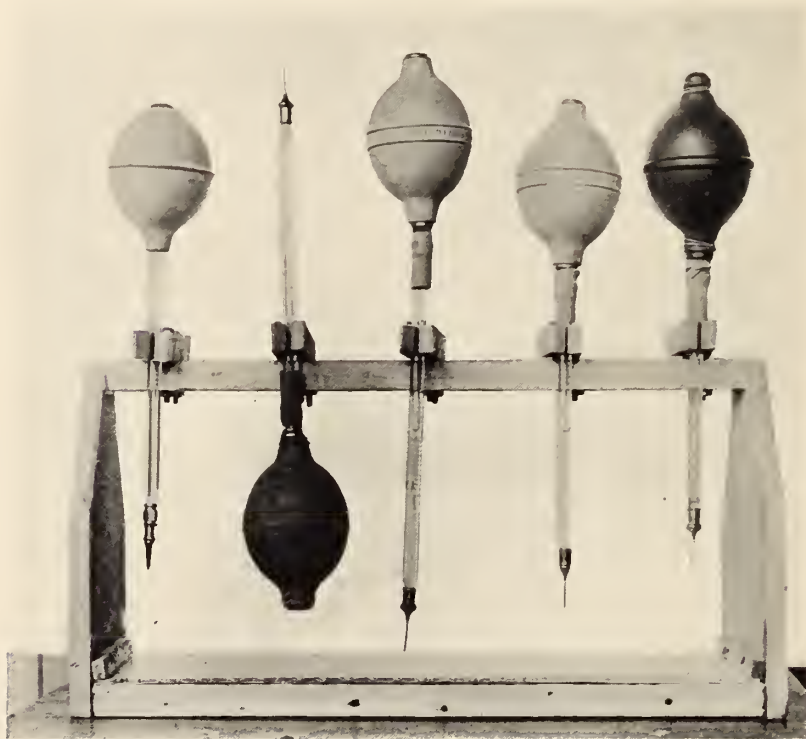


FIG. 4

proved equal to the requirements. Its accuracy is that of the pipette itself.

The holder needs no explanation beyond the illustration. It consists simply of a number of clothespins fastened on a stand. Other purposes it may serve besides holding the syringes are also indicated in the illustrations.

A CONTRIBUTION TO THE BOTANY OF THE ORGANISM OF BLASTOMYCOSIS.*

ROSS C. WHITMAN.

(From the Department of Pathology, University of Colorado, Denver, Colorado.)

Through the kindness of Dr. Charles A. Powers, I have been able during the past year to study the lesions from a fatal case of systemic blastomycosis, and certain of my observations seem to have an important bearing on the disputed questions in regard to botanical relations of the organism. Briefly, I believe that I have observed the ascus of the organism, and that I have thus been enabled to determine the true botanical position of the plant within fairly well defined limits—limits which, it is hoped, will be still more sharply defined by the results of further observations now in progress.

A brief review of the history of the controversy will best serve to define the questions at issue.

The first authentic case of blastomycosis was studied and reported independently by Busse¹ and by Buschke² in 1894-6. In 1898 Gilchrist³ and Stokes reported the first American case. The organisms in these cases presented certain differences, and are probably not identical. That of Busse and Buschke has been assigned to the saccharomycetes by De Beurmann and Gougerot.⁴ Gilchrist and Stokes called their organism blastomyces because it was found in the lesions as a yeastlike cell which quite obviously multiplied by budding. This term, which has come to be almost universally employed in this connection, was offered only as a temporary makeshift by Gilchrist and Stokes, and is highly objectionable because the presence or absence of budding forms has little or no biological significance, such forms being found widely distributed among the fungi. It has long been abandoned by mycologists, in any save a purely morphological sense; the yeasts, which

* Received for publication, June 2, 1913.

¹ *Centralbl. f. Bakteriol.*, 1896, 16, p. 175; *Virch. Arch.*, 1895, 140, p. 23; *Ibid.*, 1896, 144, p. 360.

² *Volkmann's Samml. klin. Vortr.*, N. F., 1898, No. 218 (Chir. No. 64, p. 1081); *Arch. f. Dermat. u. Syph.*, 1899, 47, p. 261.

³ *Jour. Exper. Med.*, 1898, 3, p. 53.

⁴ *Les nouvelles mycoses*, Paris, 1912.

are perhaps especially characterized by this mode of reproduction, being now designated as *saccharomyces*—a designation of course, which is based on quite another property.

As indicating that the term *blastomycosis* was regarded as inaccurate (and therefore undesirable), even very early in the discussion of the disease, Bassoe¹ may be cited:

"I have used the term *blastomycosis* with considerable misgiving, as I am aware that there is a tendency among botanists to designate as *blastomycetes* the budding forms of any fungi which otherwise may not at all belong together. For the present the term *blastomycosis* is used for lack of a better one, and in order to emphasize the fact, so important from a medical standpoint, that we are dealing here with an infection by organisms which bud in the tissues of the human body."

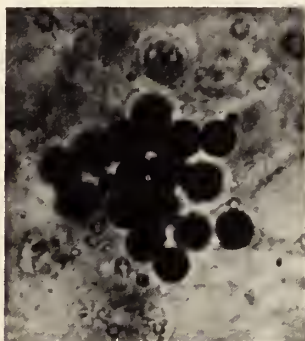


FIG. 1.—High magnification. Half-grown spores, showing possible budding.

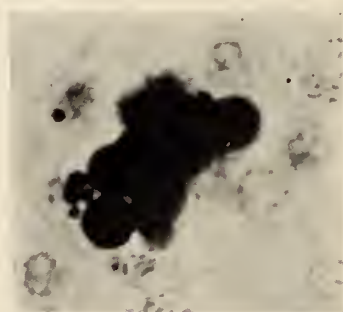


FIG. 2.—High magnification. Half-grown spores, showing possible budding.

Granted that the budding process has no biological significance, it is difficult to understand in what its importance consists. Any morphological feature is important so long as it remains an individual and peculiar characteristic. Its importance diminishes from the diagnostic standpoint in proportion as it becomes common to a wide variety of organisms. Indeed there is apparently ample ground for the view that the term *blastomycosis* really includes a wide variety of organisms. Cultures from different cases present considerable variations in morphology and cultural characteristics, and certain cases are marked by the great abundance on the one hand, or absence on the other, of budding forms. The well known fact that certain cases recover rapidly under potassium iodide, while others progress obstinately in spite of it, points in the same

¹ *Jour. Infect. Dis.*, 1906, 3, p. 91.

direction. The differentiation of these types (if indeed differences exist) is bound to remain a practically unsolved problem until the way is found to study these plants in their natural habitat. According to De Beurmann and Gougerot,¹ the immuno-diagnostic reactions fail to differentiate some of these conditions.

In 1901 Ricketts² made a detailed study of an extensive series of cases, and came to the conclusion that the organism is an oïdium, basing his opinion on the abundant formation of spores of the oïdium type (conidea) in cultures; while Ophüls³ preferred the designation coccidioides or coccidioidal oïdiummyces. Ophüls believes,



FIG. 3.—High magnification. Group of fully developed spores.

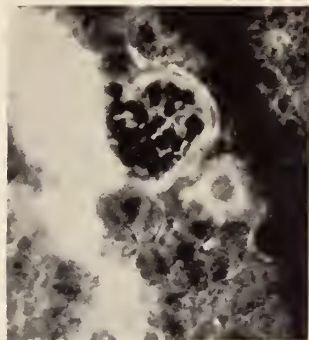


FIG. 4.—Medium magnification (about 700X). Ascus with spores some of which are just escaping from the sac.

on somewhat inadequate grounds, that the disease observed by him in California is not identical with the "Chicago disease." However this may be, these two terms seem to be slowly replacing the older designation in this country. But multiplication by means of coccidia is not confined to Linck's oïdium. On the contrary, like the formation of yeastlike bodies, it is widely distributed among the molds. For this reason both Ricketts and Ophüls regarded their suggestions as anything but final. The discovery of a higher type of fruit organ (sporangium, ascus) would serve at once to remove the plant from the oïdium group, and to place it finally and definitely in its proper position.

¹ *Loc. cit.*

² *Jour. Med. Res.*, 1901, 1, p. 373.

³ *Jour. Am. Med. Assn.*, 1905, 45, p. 1291.

In 1909 De Beurmann and Gougerot¹ made an exhaustive comparative study of the "blastomycetic" organisms and arrived at the following classification, based on the mycologic system of Van Tieghem. According to this system the exoascos are a family of the discomycetes. De Beurmann and Gougerot say: "Under the term exoascos we include all the mycoses due to parasites known to belong to the botanical group exoascos, or which can be assimilated to it" (by analogy). The following grouping of cases is thus arrived at:

1. (a) *Saccharomyces*, typical yeasts, and *Atelosaccharomyces*, yeasts in which ascus production has not been observed.—This group includes a number of isolated cases, the most important of which, for our purpose, is the case of Busse and Buschke, already cited, the organism of which was assigned to the yeasts by Vuillemain and Guiguén, and called cryptococcus.

(b) *Parasaccharomyces*.—An organism closely related to the yeasts, as the name implies, but presenting characteristics indicating that it is a transitional form between the yeasts and endomyces.

2. *Zymonema*.—A new genus proposed by De Beurmann and Gougerot. The name indicates that the plant shows mixed characters, namely, yeast forms and mycelial threads. It includes the cases variously designated as blastomycosis, oïdiomycosis, coccidioidosis, and Chicago disease. These cases cannot be properly assigned to either the first or the third group.

3. *Endomyces*.—The organism of thrush, formerly called *oïdium albicans*, which has now been definitely shown to be an endomyces, not an oïdium.

According to De Beurmann and Gougerot this classification is final with the single exception of the proposed new second group, *Zymonema*. The last is frankly placed here by analogy, in the expectation that the character of the ascus, when discovered, will justify its remaining in this place.

Cultures from the case placed at my disposal by Dr. Emery have been grown on various media, and have been observed from time to time for about one year, without disclosing more than what numerous other observers have recorded. One finds the mycelial

¹ *La tribune médicale*, 1909, 42, pp. 501, 517, also *loc. cit.*

threads septate and branching at irregular intervals; chlamydospores; conidiospores, forming either at the ends of the hyphae, or in continuity, the conidiospores in chains and separated by septa, in which case they represent the oïdium type of spore formation. The conidiospores are at first elongated. Later they become more and more spherical, and are finally set free to become, apparently, the yeast cell bodies from which the organism derived its earliest name. It is worth noting that the conidium-bearing hyphae are larger and denser than the vegetative threads.

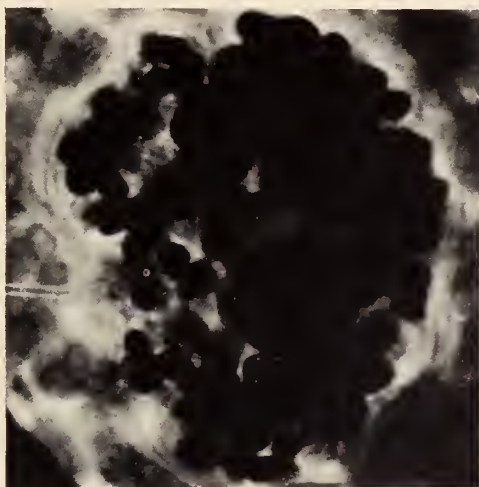


FIG. 5.—High magnification (about 3500 \times). Fully developed ascus with spores. The sac appears as a refractile membrane, which is not in perfect focus in certain regions.

Irons and Graham¹ observed in unstained preparations certain globular structures with short pedicles 6–8 microns in diameter. These, and some “terminal conidia” often contained several highly refractile spherical bodies, 0.5–1 micron in diameter. The authors suggest that these were perhaps ascospores, but there is no mention of any effort made to ascertain whether this was actually the case. I have observed similar bodies, but was unsuccessful in my attempts to find them in stained preparations.

By far the most interesting and important findings were in the tissues. For the present purpose it will not be necessary to enter

¹ *Jour. Infect. Dis.*, 1906, 3, p. 665.

into a detailed description of the lesions, and I shall confine myself to a discussion only of those points which bear on the present issue. The organism as found within the tissues is a spherical body varying in diameter from 3 or 4 to about 60 microns. All the smaller bodies and some of the largest are strongly gram-positive. The smaller bodies are spherical or slightly reniform, and do not possess a capsule. They are found lying free in the lymph spaces, and occasionally in capillaries. Sometimes one or more small bodies are found within a cell, apparently a macrophage. They occur singly or in groups numbering up to 15, 20, or more. In the larger groups

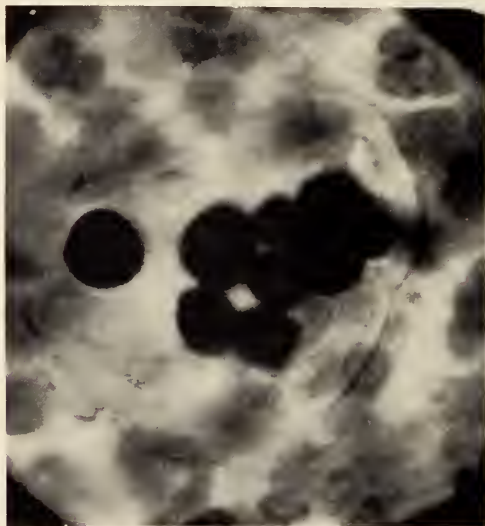


FIG. 6.—Same as Fig. 5, except the sac is occupied only by a few partly grown spores and leukocytes.

the size of the individual bodies varies within very wide limits, from the smallest to the largest sizes being observed in the same group. Budding forms, clearly recognizable as such, are exceedingly rare.

As the bodies attain full size a capsule appears about certain of them, consisting of a dense, non-staining membrane about 10 microns thick. The greater number of these membranes appear to be perfectly homogeneous, but in a certain number indications of an imperfect and somewhat irregular lamination can be made out.

Within the capsule the body may be either homogeneous, like

the non-encapsulated forms, or, more rarely, condensations of chromatin at two or three points can be made out, with a thinning of the same in the intervening areas. The points of condensation are approximately the same size as the spores next to be described. The intervening steps between this stage and the fully developed ripe ascus have not been observed. The ascus consists of a capsule as above described, round or slightly oval, within which are found a large number of monocellular spores up to 50 or more, approximately 7 microns in diameter, round or slightly reniform, and lying closely packed in the sac. It is obviously impossible to make an

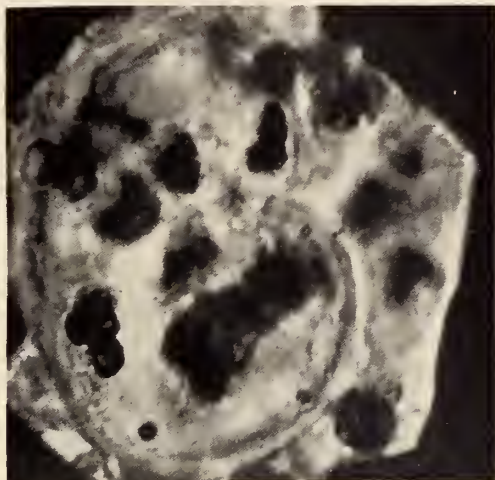


FIG. 7.—Same as Fig. 5. Sac empty save for a few leukocytes, not in perfect focus, and a few red cells.

accurate count of the number in a cross-section of the ascus, or to determine whether the number is the same in all cases, when the number is so large. This point cannot be determined until a culture medium is found, on which the plant will grow freely and produce its fruit in characteristic fashion. It is curious that no one seems to have tried growing the plant in the natural habitat of the fungi to which it appears to be most closely related. In all probability it does not belong to the saprophytic molds in the series, since in this case it might be expected to grow readily to full maturity on the various laboratory media. It is my intention to observe its behavior during the coming summer as a parasite on the

various plants known to be subject to disease caused by similar organisms. Should these experiments prove successful, it may afford a final solution of the problem of its true botanical position, and at the same time give us the means for arriving at an adequate prophylaxis.

The further development of the asci can be traced in the tissues without difficulty. At a certain stage the ascus ruptures, and the spores are discharged into the neighboring tissue. Here they lie free for a time, and enlarge, but with unequal rapidity, so that, as already noted, groups of spores contain individuals of every size

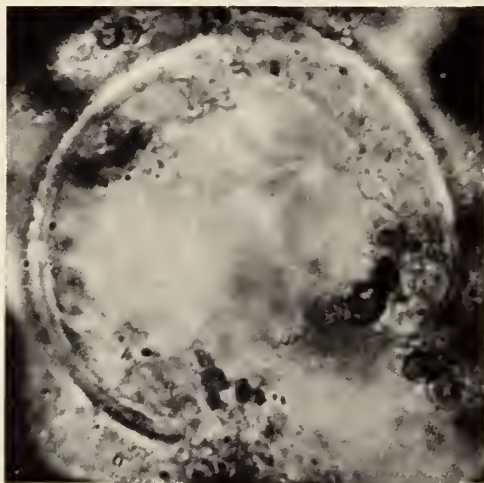


FIG. 8.—Same as Fig. 5. Sac empty.

from the smallest to the largest. Some are taken up by leukocytes (macrophages) and are carried to lymph vessels or blood capillaries, or, apparently, they may find their way to either of these destinations without being taken up. Giant cells form about them, either inside or outside the vessel. The empty capsule which remains is also attacked by leukocytes, and is no doubt ultimately destroyed by them.

I may add here that I have also observed in this case, so far as I know for the first time, the production of mycelium within the tissues. In only one field have I encountered an unmistakable mass of mycelium somewhat resembling the "sulfur granule" of

actinomycosis. In several instances, however, I have observed spores from which distinct mycelial threads can be traced for a short distance.

As already stated, De Beurmann and Gougerot have followed the mycologic system of Van Tieghem, but I am advised that the much later system of Engler and Prantl¹ is now generally accepted by mycologists as authoritative, and I therefore follow the latter even at the risk of further complicating the question.

Table I, which is based on Engler and Prantl, gives the type characters of the various organisms with which the blastomyces may possibly belong. Certain differences in detail between the two systems of classification will be discussed more fully later.

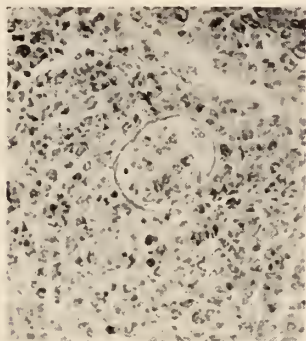


FIG. 9.—Low magnification (about 700X). Ascus empty of spores, and in course of invasion by leukocytes.

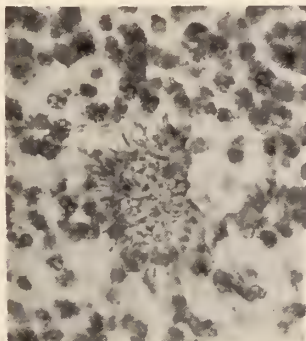


FIG. 10.—Low magnification (about 700X). Small mass of tangled mycelial threads.

This classification of Engler and Prantl shows certain important differences from that used by De Beurmann and Gougerot. The Exoascaceae are not included under the discomycetes, nor in the same family with the Protoascineae, which includes the saccharomyces and endomyces. In accordance with this classification the organism may be a variety of either of two species, which are, moreover, rather widely separated. The definition of the species *Monascus* of the genus *Monascaceae*, of the family Hemiascineae fits the findings save for the perithecium consisting of tangled mycelial threads. But the “irregular and imperfect lamination” already referred to may prove to be such a perithecium. On the

¹ *Natürliche Pflanzenfamilien*, Teil 1, Abteilung 1, p. 142, Leipzig, 1897.

possible a definite settlement of this question. If it proves to be a variety of *Taphria*, the classification proposed by De Beurmann and Gougerot will have been fully confirmed. In this connection it may be noted that occasionally one finds groups of the smallest, and presumably therefore only recently liberated spores, broken up into still smaller bodies, sometimes with tiny processes. Such a group of budding (?) spores suggests very strongly the appearance of a splash of water which has been thrown forcibly on the floor. If I am right in interpreting these groups as instances of multiplication of the spores by budding, we have an added argument for including the organism with the Taphriace.

CONCLUSIONS.

1. The organism of blastomycosis is an ascus-producing fungus. The ascus when ripe contains a large number (40 or more) of monocellular spores. It is not yet determined whether the number is or is not constant.

2. On the basis of present knowledge the plant may belong either to the Hemiasceae genus *Monascaceae*, or to the Euasceae group *Exoasceae*, species *Taphria*. In the latter case, the view of De Beurmann and Gougerot becomes established, except in the matter of its close relationship of saccharomyces and endomyces, a difference which depends on the system of classification employed.

3. The name suggested by De Beurmann and Gougerot, namely, *Zymonema*, may be accepted, unless further observation should result in identifying the organism with some older member of one or the other orders above mentioned.

4. There is reason to hope that a study of the behavior of the organism under the conditions of life known to be natural to other members of the above groups may afford important and even complete information regarding its botanical position.

5. The final solution of the questions involved would render possible the recognition of the mold in its natural habitat as a plant pathogenic for man and certain animals, and would thus establish prophylaxis against the disease.

CONCERNING NATURAL HEMOLYSINS IN RABBIT SERUM.*

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During the course of some work on the site of formation of immune hemolysins, the results of which will be published in a later communication, it was necessary to determine whether or not the serum of the animal about to be immunized contained any natural hemolytic amboceptors to the erythrocytes which were to be injected.

Rabbits, immunized with sheep's corpuscles, were used. Before starting the injections, the sera of the rabbits were tested for natural anti-sheep hemolysins and in 16 of the 18 used, or 88 per cent, their presence was demonstrated.

This result was unexpected, and accordingly we titrated an additional 32 normal rabbit sera to determine if such a large percentage were accidental. The result is shown in Table 1.

A fairly minute search of literature failed to give us any reference to observations along this line. Bordet¹ states that "it is a general rule, applicable to all sera, that the power to destroy red blood corpuscles disappears on heating to 55° C." Subsequently he says: "Guinea-pig serum and rabbit serum have both a very slight destructive effect on hen and human corpuscles. Rabbit serum, however, is much more active against guinea-pig corpuscles, but is almost entirely without effect on rat corpuscles Normal rabbit serum contains a sensitizer for goat corpuscles." Ehrlich² observes that rabbit serum is often hemolytic to goat red blood corpuscles and that it contains a very variable amount of hemolysin for guinea-pig blood.

After observing the almost constant occurrence of natural anti-sheep hemolysin in rabbit serum, we decided it would be interesting to determine if it contained native hemolytic amboceptors for the

* Received for publication May 14, 1913.

¹ *Studies in Immunity* (Wiley & Sons).

² *Studies in Immunity* (Wiley & Sons).

erythrocytes of the following vertebrates; chicken, dog, goat, guinea-pig, hog, man, ox, and white rat.

Preparation of materials.—The rabbits were bled from an ear and the serum separated by centrifugalization from the clot after one hour's standing. The sera were kept in sterile glass ampoules. Before testing, the serum was inactivated by heating to $55^{\circ}\text{C}.$, for 30 minutes, in order to destroy any anticomplementary substances, as well as native complement. This inactivated serum was then diluted with 9 parts of salt solution (0.85 per cent) and used in the following amounts 0.05 c.c., 0.2 c.c., 0.4 c.c., 0.8 c.c., 1 c.c., and 2 c.c., corresponding respectively to 0.005, 0.02, 0.04, 0.08, 0.1, and 0.2 c.c. of the undiluted serum. Complement was furnished by fresh guinea-pig serum, using 1 c.c. of a 5 per cent dilution in normal saline. The corpuscles were used in dose of 1 c.c. of a 2.5 per cent suspension in all the tests, with the exception of the chicken cells, which was a 2 per cent, and the white rat, which was a 1.7 per cent suspension.

Technic.—In tube 1, was placed 0.05 c.c. of the 1:10 inactivated rabbit serum; in tube 2, 0.2 c.c.; in tube 3, 0.4 c.c.; in tube 4, 0.8 c.c.; in tube 5, 1 c.c.; and in tube 6, 2 c.c. Then were added 1 c.c. of the 1:20 guinea-pig complement and 1 c.c. of the corpuscle suspension and enough normal salt solution to make the total volume 4 c.c. After shaking, the tubes were placed in the incubator at $37^{\circ}\text{C}.$, for 2 hours, removed, and allowed to stand in the refrigerator over night and the results read in the morning.

Controls.—In every instance the guinea-pig serum used as complement was tested for natural hemolysin for the erythrocytes of the animal being tested. Guinea-pig serum frequently contains an appreciable amount of natural anti-sheep amboceptor but when used in dosage of 1 c.c. of 1:20 dilution, the amount of natural amboceptor for the various red cells of the other animals used was not sufficient to require removal before the serum could be used for complement. Each serum was tested in maximum dosage with the corpuscles to make sure that all native complement was rendered inactive; with several sets of reactions, specific immune hemolysins were used with the sera to make certain that none of the sera were anticomplementary. The corpuscle suspensions were always

prepared of fresh blood and controlled as to undue fragility, by suspending a dose in salt solution.

Natural anti-sheep hemolysin.—The sera obtained from 50 normal rabbits were tested with the results as shown in Table 1.

TABLE 1.

Amount of Serum c.c.	100% Hemolysis Percentage	75% Hemolysis Percentage	25% Hemolysis Percentage	0 Hemolysis Percentage
0.005.....	0	2	10	24
0.02.....	2	8	20	"
0.04.....	4	10	40	"
0.08.....	6	22	56	"
0.10.....	14	40	68	"
0.20.....	18	56	76	"

It is evident that inactivated rabbit serum contains considerable natural anti-sheep hemolysin; 38 of the 50 sera examined exhibiting it. In 2 per cent of them 0.02 c.c. of serum was sufficient to cause complete hemolysis and in 12 per cent such a small amount as 0.005 c.c. of serum caused from 25 to 75 per cent of hemolysis. It is generally recognized that some rabbits yield a higher output of immune hemolysis than others; but the difference may be largely due to this observation concerning the high content of natural hemolysin in 10 to 12 per cent of rabbits.

Natural anti-human hemolysin.—Fifty rabbit sera were tested against human erythrocytes with results as tabulated below.

TABLE 2.

Amount of Serum c.c.	100% Hemolysis Percentage	75% Hemolysis Percentage	25% Hemolysis Percentage	0 Hemolysis Percentage
0.005.....	0	0	0	80
0.02.....	0	0	8	"
0.04.....	0	0	12	"
0.08.....	0	0	12	"
0.10.....	0	2	14	"
0.20.....	0	4	20	"

Here we note that 40 of the 50 sera showed no hemolysin when used in amounts up to 0.2 c.c. and of the 10 giving hemolysis none was able to cause complete destruction, one gave 75 per cent hemolysis in an amount of 0.1 c.c., and four showed slight (25 per cent) hemolysis in doses of 0.02 c.c.

Anti-goat hemolysin.—Twenty-five rabbits were examined for natural anti-goat hemolysin.

TABLE 3.

Amount of Serum c.c.	100% Hemolysis Percentage	75% Hemolysis Percentage	25% Hemolysis Percentage	0 Hemolysis Percentage
0.005.....	0	0	4	12
0.02.....	0	4	10	"
0.04.....	0	4	44	"
0.08.....	0	8	72	"
0.10.....	0	28	76	"
0.20.....	4	52	88	"

Only three of these sera contained no hemolytic amboceptors. Of the remaining 22 which exhibited hemolysis, one showed complete hemolysis with 0.2 c.c. of serum and 13 gave 75 per cent hemolysis with the same amount. The smallest amount of serum causing any hemolysis was 0.005 c.c.

Anti-dog hemolysin.—Table 4 shows the results of examining 25 sera for anti-dog hemolysin.

TABLE 4.

Amount of Serum c.c.	100% Hemolysis Percentage	75% Hemolysis Percentage	25% Hemolysis Percentage	0 Hemolysis Percentage
0.005.....	0	0	0	28
0.02.....	0	0	4	"
0.04.....	0	8	16	"
0.08.....	0	16	48	"
0.10.....	12	32	52	"
0.20.....	32	40	72	"

Here we note that the anti-dog hemolytic amboceptors contained in normal rabbit serum are more potent than any of the others studied, but their presence is not so constant nor will such small amounts of serum cause hemolysis, it requiring at least 0.02 c.c. to cause 25 per cent hemolysis. However, in 12 per cent of the cases showing hemolysis, 0.1 c.c. of serum causes complete destruction of the dog's red blood corpuscles.

Anti-hog hemolysin.—Twenty-five sera were tested for anti-hog hemolysin (Table 5).

Twenty of these failed to show any trace of amboceptor. Of the 5 sera causing hemolysis, none gave complete hemolysis with the

TABLE 5.

Amount of Serum c.c.	100% Hemolysis Percentage	75% Hemolysis Percentage	25% Hemolysis Percentage	0 Hemolysis Percentage
0.005.....	0	0	0	80
0.02.....	0	0	0	"
0.04.....	0	0	4	"
0.08.....	0	0	8	"
0.10.....	0	0	20	"
0.20.....	0	4	20	"

largest amount of serum used and 0.04 c.c. was the smallest amount which could dissolve 25 per cent of the hog's erythrocytes.

Anti-ox hemolysin.—Table 6 gives the results of examining 25 sera for anti-ox hemolysin.

TABLE 6.

Amount of Serum c.c.	100% Hemolysis Percentage	75% Hemolysis Percentage	25% Hemolysis Percentage	0 Hemolysis Percentage
0.005.....	0	0	0	80
0.02.....	0	0	4	"
0.04.....	0	0	8	"
0.08.....	0	0	8	"
0.10.....	0	0	16	"
0.20.....	0	4	20	"

The amount of natural anti-ox hemolytic amboceptor appears to be about the same as the anti-human and the anti-hog hemolysins already commented on.

Anti-chicken hemolysin.—Twenty-five sera were tested for anti-chicken hemolytic amboceptors.

TABLE 7.

Amount of Serum c.c.	100% Hemolysis Percentage	75% Hemolysis Percentage	25% Hemolysis Percentage	0 Hemolysis Percentage
0.005.....	0	0	0	92
0.02.....	0	0	0	"
0.04.....	0	0	0	"
0.08.....	0	0	4	"
0.10.....	0	0	4	"
0.20.....	0	4	8	"

Only two sera exhibited any hemolysin. At least 0.08 c.c. of serum was necessary to give 25 per cent hemolysis and in the other case the largest amount of serum used, 0.2 c.c. caused 75 per cent hemolysis.

Anti-guinea-pig hemolysis.—Table 8 shows the examination of 25 rabbit sera for anti-guinea-pig hemolysin.

TABLE 8.

Amount of Serum c. c.	100% Hemolysis Percentage	75% Hemolysis Percentage	25% Hemolysis Percentage	0 Hemolysis Percentage
0.005	0	0	0	96
0.02	0	0	0	"
0.04	0	0	0	"
0.08	0	0	0	"
0.10	0	0	0	"
0.20	0	0	4	"

Twenty-four of the sera showed no trace of hemolysin and the only one giving hemolysis required the largest amount of serum used, 0.2 c.c., to cause a 25 per cent hemolysis.

Anti-rat hemolysin.—Twenty-five sera were tested for anti-white rat hemolysin with negative results in all cases. The white rat (*Mus norvegicus*, var albinus) is the only animal examined for which rabbit serum caused no destruction of its red blood cells.

The foregoing tables, with the accompanying remarks, give all the essential data, but it will be of interest to analyze the results more minutely and determine what were the smallest amounts of normal rabbit's serum causing 100 per cent, 75 per cent, and 25 per cent destruction of the red blood cells of the various animals investigated and what percentage they bear to the number of sera tested. These results are shown in the accompanying table.

TABLE 9.

SHOWING SMALLEST AMOUNTS OF RABBIT'S SERUM GIVING 100, 75, AND 25 PER CENT HEMOLYSIS.

Blood	100% Hemolysis	75% Hemolysis	25% Hemolysis
Chicken		0.2 c.c. (4%)	0.08 c.c. (4%)
Dog	0.1 c.c. (12%)	0.01 " (8%)	0.02 " (4%)
Goat	0.2 c.c. (4%)	0.02 " (4%)	0.005 " (4%)
Guinea-pig			0.2 " (4%)
Hog		0.2 " (4%)	0.04 " (4%)
Human		0.1 " (2%)	0.02 " (8%)
Ox		0.2 " (4%)	0.02 " (4%)
Rat—white			
Sheep	0.02 c.c. (2%)	0.005 " (2%)	0.005 " (10%)

It will be noted that as small an amount as 0.005 c.c. of rabbit serum contains enough amboceptor to cause 25 per cent hemolysis

in 10 per cent of the tests for anti-sheep hemolysin and 4 per cent of the anti-goat determinations. Also, that the smallest amount giving complete hemolysis was 0.02 c.c. in the case of sheep's corpuscles, 0.1 c.c. with dog's red blood cells, and 0.2 c.c. with the goat's erythrocytes.

In conclusion, we have arranged the various bloods under investigation in the order in which rabbit serum contains the most constant amount of natural hemolytic amboceptors for them.

TABLE 10.
SUMMARY OF NATURAL HEMOLYSINS IN NORMAL RABBIT SERUM.

Blood	Number of Sera Tested	100% Hemolysis Percentage	75% Hemolysis Percentage	25% Hemolysis Percentage	0 Hemolysis Percentage
Goat.....	25	4	52	88	12
Sheep.....	50	18	56	76	24
Dog.....	25	32	40	72	28
Human.....	50	0	4	20	80
Hog.....	25	0	4	20	80
Ox.....	25	0	4	20	80
Chicken.....	25	0	4	8	92
Guinea-pig.....	25	0	0	4	96
Rat—white.....	25	0	0	0	100

SOME IMMUNITY REACTIONS OF EDESTIN.*†

THE BIOLOGICAL REACTIONS OF THE VEGETABLE PROTEINS. III.¹

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INTRODUCTION.

The study of the immune bodies produced in response to the parenteral-introduction of pure proteins has a twofold application. First, by using a pure protein, all the usual accompanying substances present in serum, bacterial masses, etc., such as other proteins, less complex nitrogenous bodies, lipoids, fats and carbohydrates, are eliminated, and the antibodies elaborated may be considered solely as the result of a specific reaction against the protein injected. Therefore these reactions, when analyzed, afford a definite criterion for judging the theories of immunity. Second, the immunity reactions of the pure proteins may serve to establish the biological relationships of various proteins, and possibly to yield information concerning their more intimate chemical constitution and chemical relationships.

The study of proteins of animal origin offers a somewhat limited field, since of all these proteins egg albumin alone can be isolated in a state of purity sufficient for these delicate biochemical tests. The immunity reactions of this substance have been extensively investigated, but the application of the data obtained is necessarily more or less restricted, since it is impossible to prepare comparative proteins of animal origin in as pure a state.

The vegetable proteins, on the other hand, seem to present more advantageous material. Their sources are many, their kinds varied, several of them can be crystallized, and their chemical identities established by the methods developed by Osborne² and

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¹ Article I of this series, Wells and Osborne, *Jour. Infect. Dis.*, 1911, 8, p. 66; Article II, Wells and Osborne, *ibid.*, 1913, 12, p. 341.

² *The Vegetable Proteins*, Longmans, Green & Co., London, 1909.

others. Some of them, however, particularly the globulins, introduce discouraging complications, which make the prosecution of the various reactions difficult. The insolubility of the globulins in isotonic salt solution, their strong tendency to precipitate in the presence of serum, and their agglutinating action on the red blood corpuscles are some of the obstacles which are responsible for many experimental failures and for the comparatively scant attention which has been accorded them in immunity studies.

The present study began in an endeavor to obtain controlling evidence for certain other experiments in anaphylaxis which have been carried on in this laboratory for the past two years, hence no attempt has been made to extend the study to any proteins other than edestin. Gliadin has been used merely to test the specificity of the edestin reactions. Naturally, it is highly desirable to institute further controls with other similar proteins and to investigate the immunity reactions of these proteins as well. It is hoped to continue this work in the future.

The preparations used in immunological work obviously must be of the highest possible integrity. Those used in the present study consisted of one of edestin, the globulin from hemp seed (*Cannabis sativa*), and one of gliadin, the prolamins from wheat (*Triticum vulgare*), and were specially prepared by Dr. Thomas B. Osborne for the anaphylactic experiments which have been reported by Dr. H. Gideon Wells and himself. No utensils used in making these particular lots of edestin and gliadin had ever before been employed in preparing proteins, and the most scrupulous care was exercised to meet every possible objection to their mode of preparation.¹

It is unnecessary to review here the literature on the biological and immunological reactions of the vegetable proteins, inasmuch as it has been so thoroughly done by Wells and Osborne.² Further, a detailed description of the antigenic properties of these proteins has recently appeared.³ Various investigators have reported observations on the hemagglutinating properties of some of these sub-

¹ The authors desire to express here their sincere appreciation of Dr. Osborne's many kindnesses and his generosity in supplying them with these two preparations.

² *Jour. Infect. Dis.*, 1911, 8, p. 66.

³ Pick, *Handbuch der pathogenen Mikroorganismen*, Kollé und v. Wassermann, 1912, 1, p. 685.

stances, on the precipitins produced, and on their hyper-sensitive or anaphylactic reactions.

HEMAGGLUTININS.

The agglutinating action of vegetable proteins upon red blood corpuscles has been frequently observed. The crude nature of the extracts employed and the variety of methods used by the various authors make quantitative comparisons difficult. Among the few investigators who employed pure proteins are Osborne, Mendel and Harris,¹ who studied ricin (*Ricinus zanzibarensis*), and Schneider,² working with proteins of the bean (*Phaseolus*). Ricin was found to agglutinate erythrocytes even when added in small amounts and this action is particularly associated with the coagulable albumin of the castor bean. Schneider concluded that the reserve protein of the bean is probably responsible for the agglutinating action, since the action decreases as the seedling develops. Further, the hemagglutinin does not occur in extracts of the root, stems or leaves of the bean plant. These observations are confirmed by those of von Eisler and von Porthelm.³ Mendel⁴ has shown that a large number of leguminous seeds possess hemagglutinating properties and that this hemagglutination and toxic action are independent.

It is apparent that various proteins show widely varying degrees of agglutinating action and it has also been found that corpuscles from different animal species exhibit different degrees of sensitive-ness to this action.

In previous investigations it has been customary to use the proteins dissolved in hypertonic salt solutions and to employ blood-corpuscles in the form of defibrinated blood. In order to exclude any action which might be associated with serum components, the corpuscles in the present experiments were previously washed free from serum. The technic was as follows:

Experiment 82.—The edestin and gliadin solutions were made by rubbing up the dry powdered preparations in a small amount of water, then diluting and adding just enough N/10 sodium hydrate solution to produce solution and finally adding sufficient

¹ *Am. Jour. Physiol.*, 1905, 14, p. 259. ⁴ *Arch. Fisiol.*, 1909, 7, p. 168.

² *Jour. Biol. Chem.*, 1912, 11, p. 47.

³ *Ber. d. deutsch. Botan. Ges.*, 1911, 29, p. 419; *Zentralbl. f. Biochem. u. Biophysik.*, 1911, 12, p. 391.

N/1000 sodium hydrate to give the required concentration of edestin of 1:100. Solutions of 1:1000 and 1:10,000 were made by adding requisite amounts of N/1000 sodium hydrate. The erythrocytes used were sheep's corpuscles washed free from serum and made up in a 1 per cent suspension in isotonic sodium chlorid solution. Sufficient salt solution (1.35 per cent) and water were first put in the tubes to render the final volume of 4 c.c. isotonic, then 1 c.c. of the corpuscle suspension was added to each tube and varying amounts of edestin and gliadin solutions were immediately added slowly, with shaking, in order to prevent the laking action of these hypotonic solutions. Macroscopic and microscopic observations after 15 min. and 7 hrs.

TABLE 1.
HEMAGGLUTINATING ACTION OF EDESTIN AND GLIADIN.

TUBE	EDESTIN			RED CELLS 1% (c.c.)	SODIUM CHLORID SOLUTION 1.35 PER CENT (c.c.)	WATER (c.c.)	AGGLUTINATION	
	Dilution	Quantity (c.c.)	Milligrams				15 min. 37.5°	7 hrs. 2 hrs. 37.5° 5 hrs. 16.0°
1.....	1:100	1.0	10	1.0	2.0	+++++	+++++
2.....	"	0.5	5	"	"	0.5	+++++	+++++
3.....	"	0.25	25	"	"	0.75	+++	++++
4.....	"	0.1	1	"	"	0.9	++	++++
5.....	1:1000	1.0	1	"	"	+	++++
6.....	"	0.5	5	"	"	0.5	±	+++
7.....	"	0.25	0.25	"	"	0.75	0	++
8.....	"	0.1	0.1	"	"	0.9	0	+
9.....	1:10000	1.0	0.1	"	"	0	+
10.....	"	0.5	0.05	"	"	0.5	0	0
11.....	"	0.25	0.025	"	"	0.75	0	0
		<i>Gliadin</i>						
12.....	1:100	1.0	10	"	"	0	0
13.....	"	0.5	5	"	"	0.5	0	0
14.....	"	0.25	2.5	"	"	0.75	0	0
15.....	"	"	1.0	0	0

The experiment shows that edestin possesses hemagglutinating activity to a considerable degree, while gliadin, in the amounts tested, has no such property. Since edestin is non-toxic, it would seem, as has been previously observed, that the agglutinating action and toxic action are independent. Further, since washed corpuscles were employed, the action must have been between the edestin and the corpuscles, and since by using a pure preparation of edestin, and thereby excluding all other constituents of the seed, the results indicate that hemagglutination is a property of the seed protein.

In view of the observations of Raubitschek¹ that immune sera, normal sera and also peptone inhibit agglutination, it seemed desirable to test this possible antagonistic action in the case of edestin. The procedure was as follows:

¹ *Centralbl. f. Bakteriöl.*, I Orig., 1908, 45, p. 660.

Experiment 84.—The edestin solution (1:100) was made as in Experiment 82. One per cent suspension of thoroughly washed human red corpuscles. Edestin immune rabbit serum No. 19 (inactivated). Fresh active normal rabbit serum. Ten per cent Witte peptone in 0.9 per cent sodium chlorid solution. Varying amounts of 1.35 per cent sodium chlorid solution and water to make all mixtures uniform in volume (4 c.c.). Requisite amounts of sodium chlorid solution and water were put in tubes first, then serum or peptone, then corpuscle suspension, the tubes shaken and finally the edestin solution was added drop by drop with shaking. Tubes were incubated 2 hrs. at 37.5°, read, placed on ice for 20 hrs. and final reading taken. Both readings were done microscopically.

TABLE 2.
ANTI-HEMAGGLUTINATING ACTION OF SERA AND PEPTONE.

TUBE	EDESTIN 1:100 (c.c.)	HUMAN BLOOD COR- PUSCLES 1% (c.c.)	EDESTIN IMMUNE SERUM (c.c.)	NORMAL RABBIT SERUM ACTIVE (c.c.)	PEPTONE 10%* (c.c.)	SALT 1.35% (c.c.)	WATER (c.c.)	AGGLUTINATION	
								2 Hrs. 37.5° C.	22 Hrs. 16° C.
1.....	0.5†	1.0	0.1	2.0	0.4	0	0
2.....	"	"	0.05	"	0.45	0	0
3.....	"	"	0.01	"	0.49	+	+
4.....	"	"	0.005	"	0.5	+	+
5.....	"	"	0.001	"	0.5	++	++
6.....	"	"	0.0005	"	0.5	+++	+++
7.....	"	"	0.0001	"	0.5	+++	+++
8.....	"	"	0.5	"	++	++
9.....	"	"	0.1	"	0.4	+	+
10.....	"	"	0.05	"	0.45	+	+
11.....	"	"	0.01	"	0.49	+	+
12.....	"	"	0.5	"	0	0
13.....	"	"	0.25	"	0.25	±	+
14.....	"	"	0.1	"	0.4	+	+
15.....	"	"	0.9% NaCl	0.5	++++	++++
16.....	"	0.1	2.9	0	0
17.....	"	0.5	2.5	++	++
18.....	"	0.5	2.5	0	0
19.....	"	2.0	1.0	0	0

* 10 per cent in salt

† 5 milligrams

The results of the above experiment confirm those of Raubitschek.¹ Edestin immune serum in as small a quantity as 0.05 c.c. completely prevents the agglutination of red cells by five milligrams edestin, under the conditions noted. Indications of this antagonistic action are observed with even smaller amounts of immune serum. In the case of normal serum there was little inhibition. Fifty milligrams of Witte's peptone completely inhibited agglutination. It should be mentioned here that both edestin and gliadin in the concentration employed are precipitated in the presence of red cells and also by the larger amounts of serum. That the agglutinating action of edestin is independent of the mechanical sedimen-

¹ *Wien. klin. Wchnschr.*, 1909, 22, p. 1065; *ibid.*, 1909, 22, p. 1752.

tation is shown by the fact that gliadin produces a heavy precipitation but no agglutination, and that five milligrams of edestin in the presence of immune serum, while precipitating, exhibit no agglutinating activity. It would seem, therefore, that edestin possesses an affinity for red blood corpuscles from both sheep and man which results in the agglutination of the cells, but that this affinity is not as strong as is that between edestin and its immune serum or Witte's peptone.

PRECIPITINS.

The desirability of determining the precipitin reactions of the vegetable proteins has naturally presented itself to many investigators. Experiments to this end have been carried out with edestin, and, among the various reported observations, those of Obermayer and Pick¹ are particularly applicable here. These authors were able to demonstrate specific precipitins in the serum of animals which had received edestin injections. A successful technic for measuring the precipitin potency of an immune serum for edestin requires a nice adjustment of the balance between the antigen and serum. Edestin solutions of a higher concentration than 1:1000 precipitate spontaneously on standing, and immediately when brought in contact with diluted (1:10) normal serum. However, if the edestin be used in a 1:10,000 dilution (prepared as described in Experiment 82) there is no spontaneous sedimentation, and the addition of dilute normal serum (1:10) produces no precipitation.

Experiment 71.—The edestin immune serum was obtained as follows: A large rabbit was given intravenous injections of 0.050, 0.100, 0.050 and 0.150 gram edestin at intervals of 5, 6 and 1 days respectively. The animal was exsanguinated 19 days later, the serum centrifuged and inactivated by heating 15 min. at 56° C. A 1:1000 edestin solution was prepared as already described and 1 c.c. of this was added to 9 c.c. of isotonic salt solution. This solution remains clear for several hours.

The results of the experiment show that edestin immune serum gives a marked precipitate with edestin, while twice the amount of serum produces no precipitation with the same amount of gliadin. Controls with normal serum were negative with both edestin and gliadin. The outcome of this experiment would seem to hold out the promise of affording a reliable method for determining

¹ *Wien. klin. Wchnschr.*, 1904, 17, p. 265; *ibid.*, 1906, 19, p. 324.

TABLE 3.
PRECIPITIN REACTIONS.

TUBE	EDESTIN 1:10000 (c.c.)	GLIADIN 1:10000 (c.c.)	EDESTIN IMMUNE SERUM IN- ACTIVATED 1:10 (c.c.)	NORMAL RABBIT SERUM IN- ACTIVATED (c.c.)	DILUENT NaOH N/1000 (c.c.)	PRECIPITATION	
						2 Hrs. 37° C.	24 Hrs. 16° C.
1	1.0	...	1.0	++++	++++
2	1.0	...	0.5	...	0.5	++	++++
3	1.0	1.0	...	+	o
4	1.0	0.5	0.5	o	o
5	1.0	1.0	o	o
6	...	1.0	1.0	o	o
7	...	1.0	0.5	...	0.5	o	o
8	...	1.0	...	1.0	...	o	o
9	...	1.0	...	0.5	0.5	o	o
10	...	1.0	1.0	o	o
11	1.0	...	1.0	o	o
12	1.0	1.0	o	o

the presence of specific precipitins in the sera of animals immunized with vegetable proteins.

COMPLEMENT DEVIATION.

Dunbar,¹ apparently, is the only observer who has employed the complement deviation method in the differentiation of vegetable proteins. He was able to differentiate the various pollen proteins, and also to distinguish the pollen protein from proteins contained in other parts of the same plant. A search of the literature has failed to reveal a record of any similar experiments with purified vegetable proteins.

Experiment 80.—The test was carried out according to the original Wassermann method with such modifications as the conditions demanded. The usual reagents were used in one-half the quantity specified in the original procedure.

1. The antigen. 0.100 gram edestin suspended in water, 0.8 c.c. N/10 sodium hydrate added and the volume made to 100 c.c. with N/1000 sodium hydrate. One part of this solution was diluted with nine parts of 0.9 per cent sodium chlorid solution giving a 1:10,000 solution of edestin. The gliadin was made in precisely the same manner.
2. Edestin immune serum. This serum was the same as already described (Experiment 71, precipitin test). Inactivated 15 min. at 56° C.
3. Normal rabbit serum. Inactivated 15 min. at 56° C.
4. Hemolytic amboceptor. Immune serum from a rabbit immunized in the usual way with washed sheep's corpuscles. Titre, 0.075 c.c. of a 1:200 dilution.
5. Complement. Pooled, fresh serum, centrifuged, from two or more normal guinea-pigs of about 300 gms. weight. One part of serum diluted with 9 parts of isotonic sodium chlorid solution.

¹ *Ztschr. f. Immunitätsf.*, 1910, 4, p. 740.

TABLE 4.

A. COMPLEMENT DEVIATION WITH EDESTIN.

Tube	Edestin 1:10000 (c.c.)	Edestin Immune Serum 1:10 (c.c.)	Comple- ment 1:10 2 Units (c.c.)	Salt 0.9% (c.c.)	SRBC Sheep (c.c.)	1 Hr. 37° C.	On Ice Over Night
1.....	0.5	0.25	0.5	0.25	1.0	++++	++++
2.....	0.4	0.25	0.5	0.35	1.0	++++	++++
3.....	0.3	0.25	0.5	0.45	1.0	++++	++++
4.....	0.2	0.25	0.5	0.55	1.0	++++±	++++±
5.....	0.1	0.25	0.5	0.65	1.0	+++	+++
6.....	0.05	0.25	0.5	0.7	1.0	++	++
7.....	0.025	0.25	0.5	0.725	1.0	+	+
8.....	0.01	0.25	0.5	0.74	1.0	0	0
Normal Rab- bit Serum							
9.....	0.5	0.25	0.5	0.25	1.0	0	0
10.....	0.4	0.25	0.5	0.25	1.0	0	0
11.....	0.3	0.25	0.5	0.45	1.0	0	0
12.....	0.2	0.25	0.5	0.55	1.0	0	0
13.....	0.1	0.25	0.5	0.65	1.0	0	0
14.....	0.05	0.25	0.5	0.7	1.0	0	0
15.....	0.025	0.25	0.5	0.725	1.0	0	0
16.....	0.01	0.25	0.5	0.74	1.0	0	0
Controls							
17.....	1.0	0.5	1.0	0	0
18.....	1.0	1.0	RBC 0.5	++++	++++
19.....	E. Im. S. 0.5	0.5	0.5	SRBC 1.0	0	0
20.....	E. Im. S. 0.5	1.5	RBC 0.5	++++	++++
21.....	Norm. S. 0.5	0.5	0.5	SRBC 1.0	0	0
22.....	Norm. S. 0.5	1.5	RBC 0.5	++++	++++
23.....	0.5	1.0	SRBC 1.0	0	0

B. GLIADIN. COMPLEMENT DEVIATION WITH EDESTIN IMMUNE SERUM NO. 19.

Tube	Gliadin 1:10000 (c.c.)	Edestin Immune Serum 1:10 (c.c.)	Comple- ment 1:10 (c.c.)	Salt 0.9% (c.c.)	SRBC Sheep	1 Hr. 37.5	On Ice Over Night
24.....	0.5	0.25	0.5	0.25	1.0	0	0
25.....	0.25	0.25	0.5	0.5	1.0	0	0
26.....	0.1	0.25	0.5	0.65	1.0	0	0
27.....	0.5	0.25	0.5	0.7	1.0	0	0
28.....	0.025	0.25	0.5	0.725	1.0	0	0
29.....	0.01	0.25	0.5	0.74	1.0	0	0
Normal Rab- bit Serum							
30.....	0.5	0.25	0.5	0.25	1.0	0	0
31.....	0.25	0.25	0.5	0.5	1.0	0	0
32.....	0.1	0.25	0.5	0.65	1.0	0	0
33.....	0.05	0.25	0.5	0.7	1.0	0	0
34.....	0.025	0.25	0.5	0.725	1.0	0	0
35.....	0.01	0.25	0.5	0.74	1.0	0	0
Controls							
36.....	0.5	0.5	0.5	1.0	0	0
37.....	0.5	1.5	RBC 0.5	++++	++++

++++ complete inhibition—no hemolysis.

+++ nearly complete inhibition.

++ some inhibition.

+ slight inhibition.

± trace of inhibition.

0 no inhibition—complete hemolysis.

RBC red blood corpuscles.

SRBC sensitized red blood corpuscles.

6. Sheep's corpuscles. Freshly obtained and thoroughly washed sheep's corpuscles, 5 per cent suspension in isotonic salt solution.
7. Sensitized sheep's corpuscles. A mixture of the 5 per cent suspension of sheep cells, hemolytic amboceptor serum and isotonic sodium chlorid solution was made so that 1 c.c. of the mixture contained 0.5 c.c. of the corpuscle suspension and two units of amboceptor. The mixture was incubated one-half hour at 37.5° and then added to the proper tubes as indicated in the table.

The hemolytic amboceptor and complement were always titrated just previous to the actual test.

The antigen-edestin immune serum and antigen-normal serum mixtures, the anti-complementary, and hemolytic controls were incubated for 1 hr. in the water bath at 37.5°, the sensitized corpuscles then added and the tubes again incubated. Readings were taken at the end of 2 hrs. incubation, the tubes placed in the ice-box over night and a final reading taken.

The results obtained by the above method are sharp and clear. Both edestin and gliadin in three times the amounts used were neither anticomplementary nor hemolytic. Minute amounts of edestin in the presence of edestin immune serum completely bound complement, while a larger amount of gliadin showed no such action. The experiment, therefore, shows that an antigen consisting solely of a pure protein can absorb amboceptor in the serum of a rabbit immunized with that particular protein, and that this combination can deviate complement. The negative action of gliadin with edestin immune serum suggests that this mechanism is specific, but it will be necessary to include other vegetable proteins and other vegetable protein anti-sera before the actual specificity of the reaction can be established.

ANAPHYLAXIS.

The vast literature on anaphylaxis contains few references to the application of this method to the study and differentiation of vegetable proteins. Among these few references, which include observations on sensitization with watery and saline vegetable extracts, there are only two investigations which have to do with isolated proteins. Rosenau and Anderson¹ employed preparations of edestin and excelsin, but failed to obtain any significant results. Several factors were responsible for this failure. They used solutions of these proteins made in 10 per cent sodium chlorid, the intoxicating doses were insufficient and were given subcutane-

¹ *Bull. No. 45, Hyg. Lab., U.S. Pub. Health and Mar.-Hosp. Serv., Wash., 1908, p. 13.*

ously. The work of Wells and Osborne represents the first and only systematic study of pure vegetable proteins as to their anaphylactic reactions. Their preparations were as pure as can be obtained by present methods and were used for the injections dissolved in one-tenth per cent sodium hydrate, thus insuring an adequate intoxicating dose of the protein and obviating the antagonistic action of the hypertonic content of sodium chlorid. All injections were made into the peritoneal cavity. The authors state that because of the tendency of vegetable proteins to cause agglutination of erythrocytes, and because their proteins were in alkaline solution, no intravascular injections were made. Unusual care was taken to control all experimental factors. They used the following preparations: globulin from the castor bean, flax seed, and squash seed; edestin from hemp seed, excelsin from the Brazil nut; proteins from the cocoanut, legumin and vicilin from peas, legumin from the vetch, vignin from the cowpea, glycinin from soy-bean, gliadin from wheat flour and rye, hordein from barley flour, and zein from maize. It was found that all these proteins cause typical anaphylaxis reactions in sensitized animals. The minimum doses which produce sensitization and the time of incubation are about the same as with animal proteins, but as a rule the symptoms are of somewhat slower onset and less stormy course than are those obtained with foreign sera, and the minimum intoxicating doses are larger. There are also considerable differences in the toxicity of the several proteins to sensitized animals. The most toxic proteins, as measured by the frequency of severe and fatal reactions, usually caused death when given in 0.1 gm. doses to properly sensitized animals. Edestin, it is interesting to note in the present connection, caused the least severe reactions of any of the proteins. Marked specificity of reaction was shown, within certain limits, by the vegetable proteins employed.

In the present study the same two preparations of edestin and gliadin used by Wells and Osborne were employed. They were dissolved in water with just enough N/10 sodium hydrate to effect solution and therefore were somewhat less alkaline than the solutions of these authors. Sensitizing injections were always intraperitoneal

but the intoxicating doses were always given intravenously. It was found that at least 40 or 60 milligrams of these proteins in faintly alkalin solution could be injected into the jugular vein of 200-gm. guinea-pigs without causing any appreciable symptoms. Whatever hemagglutinating action these solutions exerted in no way interfered with or complicated the hypersensitive reaction. Guinea-pigs of about 200 gms. in weight were always used unless otherwise stated. All glass syringes were used, one set with needles for edestin and another for gliadin. These were thoroughly cleansed and dried before use.

Sensitizing and intoxicating doses.—Wells and Osborne found that five ten-thousandth milligram (0.000005 gm.) of squash globulin effectively sensitized a guinea-pig, while one ten-thousandth milligram (0.000001 gm.) of edestin rendered a pig sufficiently sensitive to react with moderate symptoms to an intoxicating dose of 0.1 gm. edestin. The intoxicating power, however, is much less than that of egg albumin, serum proteins, etc., for the minimum intoxicating dose of their preparation of edestin was two to five milligrams, while severe reactions were not obtained with less than 20 to 40 milligrams. The intraperitoneal route of administering the intoxicating dose undoubtedly is accountable for the comparatively large amounts required to produce severe or fatal reactions. Edestin and gliadin are precipitated immediately when brought in contact with serum or tissues, hence their absorption from the peritoneal cavity must be too slow to produce the typical fulminating intoxication unless given in comparatively large amounts.

Experiment 73.—The solutions used for the sensitizing injections were made as follows: One gram edestin was suspended in a small amount of water, enough N/10 sodium hydrate added to effect solution and the volume then made up to 1 litre with N/1000 sodium hydrate. Further solutions to contain 0.1, 0.01, 0.001 and 0.0001 milligrams in 1 c.c. were made from this original solution by diluting the required amount of the 1:1000 solution to 1 litre with N/1000 sodium hydrate. The dilutions were always made in this large volume to reduce the factor of error in weighing and diluting. All dilutions were made in volumetric flasks with the N/1000 sodium hydrate at the required temperature. The indicated amounts of edestin were injected in 1 c.c. volume. The intoxicating solutions were usually made up to contain 0.020 gram edestin or gliadin in 1 c.c. Sensitizing dose, intraperitoneally, intoxicating dose, intravenously.

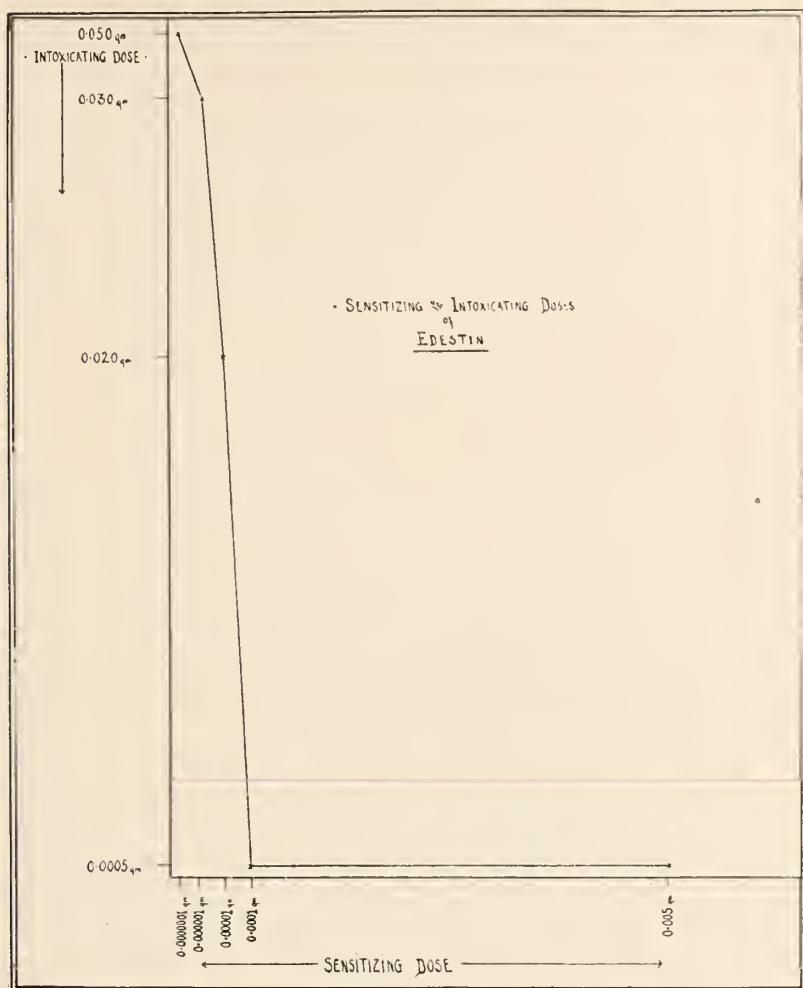
TABLE 5.

TITRATION OF SENSITIZING AND INTOXICATING DOSES OF EDESTIN.

Pig	SENSITIZING DOSE		INTERVAL (Days)	INTOXICATING DOSE		SYMPTOMS	AUTOPSY	
	Amount (Milligrams)	Volume (c.c.)		Amount (Milligrams)	Volume (c.c.)			
Series A	723.....	0.001	1.0	21	20	1.0	+ R
	732.....	0.001	1.0	21	30	1.5	+ R
	733.....	0.001	1.0	21	40	2.0	+ R
	794.....	5.0	1.0	21	30	1.5	+++R
Series B	810.....	0.0001	1.0	21	40	2.0	++R
	811.....	0.0001	1.0	21	50	2.5	+++D 4'	Typical
	807.....	0.0005	1.0	21	50	2.5	+++D 2'30"	Typical
	734.....	0.001	1.0	21	20	1.0	++ R
	730.....	0.001	1.0	21	30	1.5	+++D 3'30"	Typical
	728.....	0.001	1.0	21	40	2.0	+++D 4'30"	Typical
	731.....	0.001	1.0	21	60	3.0	+++D 3'	Typical
	738.....	0.01	1.0	21	20	1.0	+++D 3'30"	Typical
Series C	756.....	0.1	1.0	21	0.1	1.0	++ R
	755.....	0.1	1.0	21	0.25	2.5	++ R
	750.....	0.1	1.0	21	0.5	5.0	+++D 2'	Typical
	752.....	0.1	1.0	21	0.5	0.5	+++D 5'30"	Typical
Series D	757.....	0.1	1.0	21	1.0	1.0	+++D 6'	Typical
	753.....	0.1	1.0	21	5.0	0.5	+++D 4'	Typical
	754.....	0.1	1.0	21	10	1.0	+++D 4'30"	Typical
Series E	788.....	5.0	1.0	21	0.1	1.0	++ R
	784.....	5.0	1.0	21	0.5	1.0	+++D 3'30"	Typical
	789.....	5.0	1.0	21	30	1.5	+++D 4'	Typical

This experiment seems to establish the minimum sensitizing dose of edestin rendering subsequent fatal intoxication possible as one ten-thousandth milligram, and the minimum fatal intoxicating dose as one-half milligram (0.0005 gm.). Apparently ascending amounts of edestin, within certain limits, induce ascending degrees of sensitization, since 50 milligrams (0.050 gm.) edestin were required to intoxicate the animals sensitized with one ten-thousandth milligram (0.0000001 gm.) while one-half milligram (0.0005 gm.) was the minimum dose for pigs sensitized with one-tenth to five milligrams (0.0001 to 0.005 gm.) edestin. In the present investigation it has been found that guinea-pigs react uniformly and that, when suitable sensitizing and intoxicating doses of edestin are used and the intoxicating injections given intravenously, fatal reactions take place in 100 per cent of the sensitive animals. The minimum fatal intoxicating dose, when given intravenously, is one-fortieth to one-eightieth of the fatal intraperitoneal dose reported by Wells and Osborne.

TABLE 6.



In Series A an interesting point developed. By mistake some edestin was used for the intoxicating solutions which had been dried for four hours at 103° . Although the pigs in this series developed more or less marked symptoms, none died, while pigs similarly sensitized in Series B and E reacted fatally when unheated edestin was used.

Specificity.—Wells and Osborne state that animals sensitized with edestin possessed no sensitiveness to their preparations of

castor bean globulin and squash seed globulin. In the present study animals sensitized with edestin were tested with squash seed globulin, castor bean globulin, flaxseed globulin, and corylin, a globulin from the hazelnut. The three first named globulins were suggested by Dr. Osborne as being chemically and physically more nearly like edestin than any other known vegetable proteins.

Experiment 87.—The globulins were dissolved in the manner already described for edestin, and all gave clear solutions. The squash seed globulin required about four times the amount of alkali used to dissolve the other globulins, as this preparation contained a larger proportion of the insoluble form of the globulin than did the other preparations.

TABLE 7.
SPECIFICITY OF EDESTIN SENSITIZATION.

Pig	Sensitizing Dose Edestin (Milligrams)	Interval (Days)	Intoxicating Dose Globulin (Milligrams)	Symptoms	Autopsy	Interval (Hrs.)	Second Intoxicating Dose Edestin (Milligrams)	Symptoms	Autopsy
760.	5.0	41	40 gliadin	OR
759.	5.0	41	60 "	OR
775.	5.0	45	40 hazelnut	OR
762.	5.0	45	60 "	OR	48	1.0	+++D 6'30"	Typical
769.	5.0	45	40 squash seed	OD in night
773.	5.0	45	60 "	OD in night
783.	5.0	45	40 cotton seed	OR	48	1.0	+++D 3'	Typical
761.	5.0	45	60 "	OR
778.	5.0	45	20 flaxseed	++R	48	1.0	++R
777.	5.0	45	60 "	+++D 3'30"	Typical
820.	60 "	OR

Pigs 769 and 773 showed no anaphylactic symptoms but died within 12 hours. Death was probably caused by the greater alkalinity of the squash seed globulin solution. However, the small number of animals used permits of no definite conclusions. Apparently edestin fails to sensitize animals to globulins from the squash seed, castor bean and the hazelnut. Two animals sensitized with edestin reacted with typical anaphylactic symptoms, one fatally, to flaxseed globulin.¹

Inheritance of sensitiveness.—It is known that guinea-pigs born of a mother previously sensitized to a given protein inherit a certain degree of sensitiveness to that protein. The following observation is recorded here in order to illustrate this point and to show that this rule holds good in the case of edestin.

¹ This cross-reaction is merely recorded here since the specificity of the anaphylactic reactions in the case of pure vegetable proteins is being systematically investigated by Wells and Osborne.

Experiment 62.—An adult female pig received intraperitoneal injections of 0.005, 0.010, and 0.015 gm. edestin on Aug. 20, 24, and 31 respectively, being pregnant at the time. The mother's sensitiveness was shown by the fact that seven other pigs sensitized with the same amounts at the same time all reacted fatally to 0.005 gm. or more of edestin 21 days after the last sensitizing injection. On Sept. 27, 27 days after the last injection she gave birth to two young, Nos. 534-1 and 534-2. On Nov. 10, when 53 days old, they were tested as follows:

TABLE 8.

	Edestin i. v.	Symptoms	Autopsy
534-1.....	0.050 gm.	Immediate, typical Death, 2 minutes	Typical
534-2.....	0.030 gm.	Immediate, typical Recovery

Table 8 shows that both animals had inherited the mother's sensitiveness to edestin, but that they possessed it to a considerably less degree.

Passive sensitization.—Since it had been shown in the present investigation that the serum of a rabbit receiving repeated intravenous injections of edestin contains precipitating and complement deviating antibodies for edestin (Experiments 71 and 80) the endeavor was made to demonstrate the presence of an anaphylactic antibody in the same serum. It is now a well-established fact that the serum of an animal (especially the rabbit), sensitized with any protein, has the property of passively sensitizing a guinea-pig to that protein. When the proper conditions, such as the amount of serum injected, the time interval and size of the intoxicating dose, are fulfilled, the transference of sensitiveness is usually successful.

Experiments 63 and 72.—Three different edestin immune rabbit sera were used, Nos. 7, 19 and 111, the method of obtaining which has already been described. Serum No. 7 was used 24 hrs. after bleeding and was not inactivated. Sera No. 19 and 111 were inactivated by heating for 15 min. at 56°. The serum, with one exception (Pig 640), was injected intraperitoneally, and the intoxicating injection of edestin made the following day, intravenously. (See Table 9, p. 118.)

The minimum sensitizing dose, therefore, of sera No. 19 and No. 111 was between 0.05 and 0.10 c.c., although some pigs reacted fatally when passively sensitized with 0.01 and 0.025 c.c. of serum No. 111.

TABLE 9.

PASSIVE SENSITIZATION. TITRATION OF SENSITIZING DOSE OF IMMUNE SERUM.

PIG	IMMUNE SERUM		INTERVAL (HRS.)	INTOXICATING DOSE		SYMPTOMS	AUTOPSY
	Quantity (c.c.)	No.		Edestin (gms.)	Vol. (c.c.)		
640....	5.0	7	27	0.050	2.5	+++D 3'	Typical
641....	5.0	7	27	0.040	2.0	+++D 3'	"
700....	4.0	111	27½	0.040	2.0	+++D 3'15"	"
701....	2.0	111	27½	0.040	2.0	+++D 3'45"	"
702....	1.0	111	18	0.040	2.0	+++D 3'30"	"
703....	0.5	111	18	0.040	2.0	+++D 2'30"	"
704....	0.25	111	18	0.040	2.0	+++D 3'	"
717....	0.05	111	19	0.030	1.5	+++R	"
718....	0.05	111	19	0.020	2.0	+++D 4'30"	Typical
719....	0.025	111	19	0.015	1.5	+ R	"
720....	0.025	111	19	0.025	1.25	+++D 4'	Typical
721....	0.01	111	19½	0.025	1.25	+++D 3'30"	Partly inflated
724....	0.01	111	24	0.020	1.0	++ R	"
718X...	0.1	19	24	0.040	2.0	+++D 3'30"	Typical
719X...	0.05	19	24	0.040	2.0	+++D 3'30"	"
720X...	0.05	19	24	0.040	2.0	++ R	"
721X...	0.01	19	24	0.040	2.0	o R	"
722X...	0.01	19	24	0.020	1.0	o R	"

Experiment 79.—In order to compare the degree of sensitiveness produced by active and by passive sensitization the following experiment was carried out: (For titre of intoxicating dose in actively sensitized animals see Tables 5 and 6. Experiment 73). 0.1 c.c. of Serum No. 19, representing twice the minimum sensitizing dose, was injected intraperitoneally, and the intoxicating dose of edestin given intravenously on the following day.

TABLE 10.

TITRATION OF INTOXICATING DOSE OF EDESTIN IN PASSIVELY SENSITIZED ANIMALS.

PIG	EDESTIN IMMUNE SERUM No. 19 (c.c.)	INTERVAL (Hrs.)	INTOXICATING DOSE		SYMPTOMS	AUTOPSY
			Amount (gms.)	Vol. (c.c.)		
704.....	0.2	24	0.000025	0.25	o R
793.....	0.2	24	0.00005	5.0	o R
792.....	0.2	24	0.0001	1.0	+++D 3'30"	Typical
786.....	0.2	24	0.00025	0.25	+++D 4'30"	"
785.....	0.2	24	0.001	1.0	+++D 3'30"	"
787.....	0.2	24	0.0025	2.5	+++D 3'30"	"
790.....	0.2	24	0.010	1.0	+++D 3'30"	"
791.....	0.2	24	0.040	4.0	+++D 3'30"	"
789.....	0.2	24	0.015 i.p.	1.5	o R

Guinea-pigs passively sensitized with 0.1 c.c. of edestin immune serum reacted fatally to one-tenth milligram (0.0001 gram) edestin or one-fifth of the minimum amount required to intoxicate an actively sensitized pig.

The poisonous substance obtained in the hydrolysis of edestin by the method of Vaughan.—In a previous communication the authors¹ described the preparation and action of a poisonous substance obtained from the tubercle bacillus by boiling dried alcohol and ether extracted tubercle bacilli with two per cent sodium hydrate in absolute alcohol. A similar hydrolysis by the Vaughan method was performed with the preparation of edestin supplied by Dr. Osborne. The residue obtained from the alkaline alcoholic fraction, after neutralization, filtration and evaporation, was purified by redissolving four times in absolute alcohol, filtering and evaporating *in vacuo*. The final yellowish powder was exceedingly hygroscopic, soluble in water and in alcohol. Its properties and action were similar so far as tested with those already described for the poisonous product from the tubercle bacillus.

Experiment 48.—The preparation was dried over phosphorous pentoxide and sulfuric acid in a vacuum desiccator, (pressure less than 1 mm. of mercury). The poison was dissolved in isotonic salt solution and injected intravenously.

TABLE II.
TITRATION OF POISONOUS SUBSTANCE FROM EDESTIN.

GUINEA-PIG		INJECTION OF POISON			SYMPTOMS	AUTOPSY
No.	Weight	Amount (gms.)	(c.c.)	Relation to Body Weight		
695.....	215	0.0215	2.15	1:10000	+++D 3'30"	Typical
696.....	230	0.020	2.0	1:11500	+++D 3'30"	"
697.....	240	0.012	1.2	1:20000	+++D 4'45"	"
698.....	245	0.0082	0.82	1:30000	+++D 3'30"	"
699.....	245	0.0061	0.61	1:40000	+++D 4'15"	"
700.....	230	0.0046	0.46	1:50000	++ R

One part of this poisonous hydrolytic product from edestin to 40,000 parts of guinea-pig produced an acute fatal intoxication indistinguishable from anaphylactic shock. The possible relationship of this poison to the hypothetical anaphylatoxin formed in the sensitive animal and the so-called anaphylatoxin of Friedberger produced in the test tube, is discussed in the above cited communication. The chemical nature of this substance is being investigated.

Anaphylatoxin.—Friedberger observed that if fresh guinea-pig complement is allowed to act upon bacteria which have previously

¹ *Jour. Med. Research*, 1912, 26, p. 317.

been in contact with their specific immune serum, a substance is formed which produces the characteristic symptoms of anaphylactic shock. He considered that this phenomenon was due to a digestive action by the complement on the antigen-amboceptor combination and that this action *in vitro* was identical with the anaphylactic reaction *in vivo*. The hypothesis has been subjected to much criticism based upon a variety of experimental data. It has been objected that bacterial cultures and sera contain other substances, which when subjected to the action of complement might yield toxic substances. It has also been shown that complement alone, without the intervention of immune serum, is able to transform bacterial and serum protein into poisonous products, while Besredka¹ claims that the poison arises in the action of complement on the peptone of the medium on which the bacteria are grown. More recently Rosenow² has shown that the autolyzate of virulent pneumococci, when injected into guinea-pigs, calls forth a fulminating anaphylactoid reaction. A similar reaction is also produced by extracts of typhoid bacilli. In view of this confusing diversity of opinion it seemed desirable to attempt the production of the so-called anaphylatoxin from edestin by the method of Friedberger. The use of edestin as antigen would exclude the other components of bacterial masses, of sera and other protein mixtures previously used in this connection. Edestin is non-toxic in itself. Further, it is a substance which bears no near biological relationship to the constituents of guinea-pig tissues, and as edestin can be absolutely excluded from the food of guinea-pigs, it is most unlikely that they should possess any natural amboceptors for this protein. The possession of a potent immune serum, not only containing precipitating and complement deviating antibodies but also having the property of passively sensitizing guinea-pigs in small doses, supplied another desirable factor.

Preliminary experiments were carried out, using comparatively concentrated solutions of edestin and undiluted serum. A heavy precipitate immediately resulted and it was felt that this rapid precipitation might hinder the proper absorption of amboceptor by the edestin. Hence, in the later experiments (Experiments 85 and 86), the edestin and serum were employed in dilutions which

¹ *Ztschr. f. Immunitätsf.*, 1913, 16, p. 249.

² *Jour. Infect. Dis.*, 1912, 11, p. 235.

yielded precipitates only after incubation and standing at room temperature.

TABLE 12.
ANAPHYLATOXIN. A. TITRATION OF ANTIGEN.
(EXPERIMENT 64.)

PIG	EDESTIN		EDESTIN IMMUNE SERUM No. 7 (c.c.)	CONTACT 37.5° 16.0° (Hrs.)		COMPLE- MENT (c.c.)	INCUBATION TIME 37.5° (Hrs.)	SYMPTOMS
	Amount (gms.)	Vol. (c.c.)						
642	0.050	2.0	2.0	4	20	3.0	6	o
643	0.025	2.0	2.0	4	20	3.0	6	o
644	0.010	2.0	2.0	4	20	3.0	6	o
645	0.005	2.0	2.0	4	20	3.0	6	++R
650	0.005	2.0	2.0	22	..	3.0	6	++R
651	0.0025	2.0	2.0	22	..	3.0	6	++R
652	0.001	2.0	2.0	22	..	3.0	6	o
646	0.050	2.0	3.0	6	o
647	0.025	2.0	3.0	6	o
648	0.010	2.0	3.0	6	o
600	0.005	2.0	3.0	6	o
653	0.005	2.0	3.0	6	o
654	0.0025	2.0	3.0	6	o
655	0.001	2.0	3.0	6	o

TABLE 13.
ANAPHYLATOXIN. B. DETERMINATION OF INCUBATION TIME.
(EXPERIMENT 70.)

PIG	EDESTIN		EDESTIN IMMUNE SERUM No. 111 (c.c.)	CONTACT 37.5° 16.0° (Hrs.)		COMPLE- MENT (c.c.)	INCUBA- TION TIME 37.5°C (Hrs.)	SYMPTOMS	AUTOPSY
	Amount (gms.)	Vol. (c.c.)							
709	0.005	2.0	2.0	3	21	4.0	4	o	..
710	0.005	2.0	2.0	3	21	4.0	6	++R	..
711	0.005	2.0	2.0	3	21	4.0	8	OR	..
712	0.005	2.0	2.0	3	21	4.0	12	+D 8 hrs	Typical
713	0.005	2.0	4.0	4	o	..
714	0.005	2.0	4.0	6	o	..
715	0.005	2.0	4.0	8	o	..
716	0.005	2.0	4.0	12	o	..

TABLE 14.
ANAPHYLATOXIN. C. DETERMINATION OF PROPER AMOUNT OF IMMUNE SERUM.
(EXPERIMENTS 85 AND 86.)

PIG	EDESTIN		EDESTIN IMMUNE SERUM No. 19		CONTACT 37.5° 16.0° (Hrs.)		COMPLE- MENT (c.c.)	INCUBA- TION 37.5°C (Hrs.)	SYMPTOMS	AUTOPSY
	Amount (gms.)	Vol. (c.c.)	Amt. (c.c.)	Vol. (c.c.)						
800	0.001	10	1.0	10	2	22	4.0	6	o	..
801	0.002	10	0.5	5	2	22	4.0	6	++R	..
802	0.002	10	1.0	10	2	22	4.0	6	+R	..
803	0.002	10	2.5	25	2	22	4.0	6	+++R	..
816	0.005	5	3.0	30	2	22	5.0	6	+++R	..
804	0.002	10	5.0	50	2	22	5.0	6	+++D 4'30"	Typical
819	0.002	10	5.0	50	2	46	5.0	6	+++D 4'	"
817	0.002	0.2	5.0	6	o	..
818	0.005	0.5	5.0	6	o	..

Cultural tests made on the contents of the various tubes showed them to be sterile.

The above experiments show that when fresh complement is allowed to act for a definite time at 37.5° upon the precipitate obtained by mixing proper amounts of edestin and edestin immune serum, a soluble substance or substances are formed which produce the typical fatal intoxication and gross pathological appearance of true anaphylaxis, and that this substance is not formed when complement alone, under identical conditions, is allowed to act upon edestin.

CONCLUSIONS.

1. Crystallized preparations of edestin from hemp seed and gliadin from wheat flour were used, and were specially prepared for anaphylactic experiments by Dr. Thomas B. Osborne. They were dissolved in water with the addition of the smallest possible amount of sodium hydrate necessary to effect solution.

2. *Hemagglutination*.—Edestin, even in small amounts, agglutinates washed red blood corpuscles of the sheep and of man. Both edestin immune serum and peptone completely inhibit this action under the conditions noted. Gliadin, in the amounts used, exerts no agglutinating action.

3. *Precipitins*.—The serum of a rabbit which has been immunized with edestin contains a precipitating antibody for edestin, but none for gliadin in at least twice the concentration giving a positive reaction with edestin.

4. *Complement deviation*.—Edestin, in the presence of edestin immune serum, when both are used in non-anti-complementary and non-hemolytic amounts, completely binds complement. Gliadin, in the presence of edestin immune serum, fails to bind complement.

5. I. *Anaphylaxis*.—(a) Sensitizing dose, 0.0000001 gram edestin, injected intraperitoneally may be considered as the minimum sensitizing dose. Guinea-pigs sensitized with this amount react fatally when a sufficient amount of edestin (0.050 gm.) is injected intravenously after the proper interval. (b) When the sensitizing dose is one-tenth to five milligrams the intravenous injection of one-half milligram produces typical anaphylactic death in from two to six minutes.

II. *Specificity*.—Pigs sensitized to edestin fail to react to intravenous injections of gliadin, or the globulins from squash seed, the castor bean or the hazelnut. Two animals reacted positively, one fatally, when given an intravenous injection of flaxseed globulin. The fatal dose of flaxseed globulin was, however, 40 to 120 times the minimum fatal intoxicating dose of edestin.

III. *Inheritance*.—Guinea-pigs born of a mother sensitized with edestin while pregnant, inherit this sensitiveness but possess it to a somewhat less degree than does the mother.

IV. *Passive sensitization*.—The intraperitoneal injection of edestin immune rabbit serum passively sensitizes the guinea-pig. 0.05 to 0.1 c.c. of two such sera tested, rendered guinea-pigs sufficiently sensitive to react fatally to an intravenous injection of edestin on the following day. The degree of sensitiveness passively conferred appears to be somewhat greater than that induced by active sensitization.

V. *Poisonous hydrolytic product*.—When edestin is hydrolyzed by an alcoholic solution of sodium hydrate according to the method of Vaughan, a substance is formed which produces a fatal intoxication in the guinea-pig apparently identical with true anaphylactic shock. The intravenous injection of one part of this poison to 40,000 parts of guinea-pig by weight constitutes the minimum fatal dose.

VI. *Anaphylatoxin*.—When suitable amounts of edestin and edestin immune serum are allowed to remain in contact for a given length of time, a precipitate is formed, which, when washed with salt solution and mixed with fresh guinea-pig complement and incubated at body temperature, yields a substance or substances, which, when injected into a guinea-pig intravenously, produces a fatal intoxication, apparently identical in every way with the anaphylactic reaction. Fresh complement, when allowed to act under similar conditions with edestin alone, yields no poisonous substance. From edestin, therefore, by the action of immune serum and complement, under the experimental conditions noted, a toxic product is obtained which seems to correspond to the anaphylatoxin of Friedberger.

THE RÔLE OF STAPHYLOCOCCUS IN GONORRHEA.*

STUDIES ON THE GONOCOCCUS. II.

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This paper submits evidence suggesting the following tentative conclusions:

1. Many, if not all, of the gram-negative, intracellular, biscuit or coffee-bean-shaped cocci, observed in the purulent discharge in acute gonorrhea, which are regarded as gonococci and which serve as criteria of diagnosis, are not gonococci, but belong to the staphylococcus group. The provisional designation *Staphylococcus urethrae* is suggested for this coccus.¹

2. True gonococci are demonstrable with difficulty or not at all in smears of gonorrheal exudates or in preparations of tissues.

3. Gonorrhea may be due to a double infection and dependent on a microbial symbiosis.

4. Diagnosis of gonorrhea rests on cultural methods only. The old criterion, the microscopic appearances of smears, is unreliable.

The diplococci in smears of pus from acute gonorrhea (Fig. 1) are intracellular, gram-negative, biscuit or coffee-bean-shaped, clear-cut, sharply outlined, and have a marked avidity for methylene blue. Comparing these characters with those of gonococci from recent cultures (Fig. 2), we note that single cocci predominate, and that they are discrete. Many are irregularly-shaped diplococci, many are shadowy, and of indistinct contour, some are irregular in size, and nearly all take the methylene-blue stain poorly. They are always gram-negative. When gonococci are taken up by leukocytes in the guinea-pig or in the test tube, they do not at all resemble the cocci within the leukocytes in gonorrheal pus (Figs. 1 and 3), but there are cocci, namely, the staphylococci

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¹ This coccus has been described by many observers. From the literature it is apparent that it has been confused because of its protean character with many other organisms, including the true gonococcus.

urethrae, which under the same conditions appear identical with them.

In my first paper¹ I made certain observations in regard to virulent gonococci, one of which is that no colony is so young but that autolysis has begun. The lysis may be recognized by variation of size and contour, shadowy outline and faint staining. The staphylococcus in question does not possess this peculiarity. It is also noted that gonococci are not readily phagocytal. Virulent gonococci are not, as a rule, taken up, but the cocci in some old laboratory strains and killed cocci are taken up by the poly-

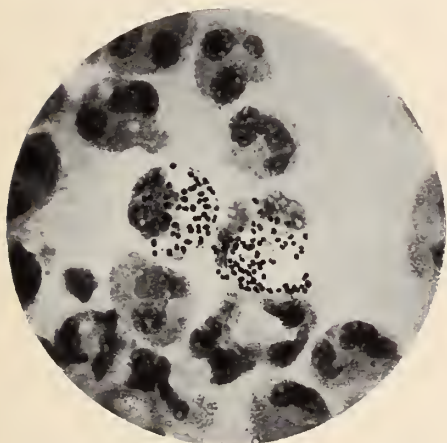


FIG. 1.—Smear from pus in acute gonorrhea. Methylene blue. $\times 1200$.

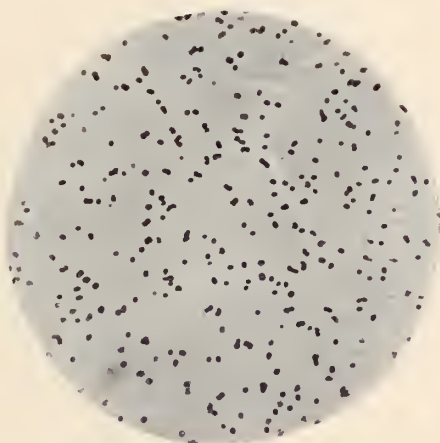


FIG. 2.—Pure gonococci from culture. Carbolfuchsin. $\times 1200$.

morphonuclear leukocytes, even in the absence of serum. All my efforts to induce free phagocytosis of live, virulent gonococci have failed, whereas the staphylococcus is readily phagocytal.

These facts lead me to question the identity and rôle of the intracellular diplococci, invariably seen in smears of gonorrheal pus, which hitherto have been accepted as gonococci.

“STAPHYLOCOCCUS URETHRAE.”

This designation will distinguish the coccus from other varieties of *Staphylococcus albus*, found elsewhere in the body, from which it differs but little. This staphylococcus has been isolated by me

¹ *Jour. Infect. Dis.*, 1913, 12, p. 93.

from 52 cases of gonorrhea, and from 20 normal urethrae. It may prove to be a habitant of normal urethrae, just as the coccus of Welch is a resident in the skin. These organisms do not lie simply in the folds and crypts of the urethral canal. If such were the case, they would be recovered more often from the urine. They lie in the cell interstices and follicles of the glands, and slight abrasion of the mucous surface is necessary to liberate them. At any rate, it is common to see colonies of staphylococci starting from a single individual or pair adhering to the border or surface of an epithelial cell. When, after 12 hours' culture on ascites agar,

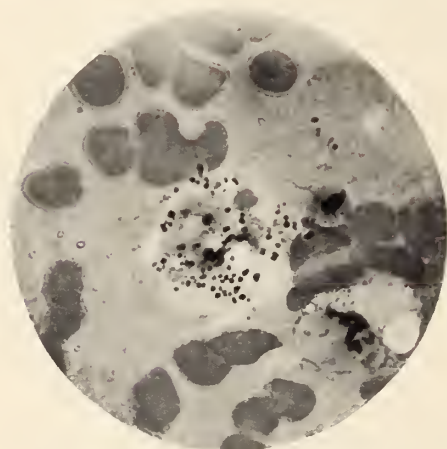


FIG. 3.—Smear from liver of guinea-pig five hours after intraperitoneal inoculation with pure culture of gonococcus. Rowmanowski. $\times 1200$.

this organism is examined in hanging drop and in stained preparations, one often observes a slight clumping, a preponderating number of biscuit or coffee-bean forms, and that some take the contrast stain and others retain the gram stain. Primary cultures and subcultures on ordinary media (broth, nutrient agar) assume the true staphylococcal type.

The variability of the staphylococcus to the gram stain is important. Suspensions

of the staphylococcus in NaCl solution show little or no change with respect to the gram stain in a month's time, whereas suspensions in gonococcus autolysate, weak pancreatin solutions, ascites and hydrocele fluids and normal serum show in progressively shorter periods of time marked gram-negative changes in the still viable cocci.

From observation of cultures and a large number of phagocytosis experiments with 15 strains of staphylococci, I have been led to believe that within the urethra during gonorrheal inflammation the staphylococcus may be gram-negative, whereas without the body, in ordinary cultures, it is gram-positive. Omitting details, the following observations have been noted:

Staphylococci taken up from NaCl suspensions by human leukocytes and incubated for a brief period are comparatively few in number, usually single, and gram-positive. Up to a certain limit the number of cocci in each phagocyte increases with the time of incubation.

Within certain limits the longer staphylococci have been in contact with serum before phagocytosis has taken place, the more numerous are the gram-negative cocci and the biscuit forms.

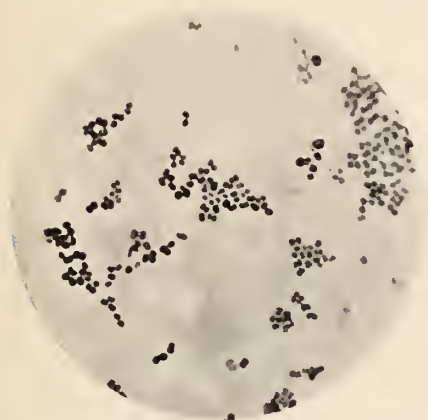


FIG. 4.—Pure culture *Staphylococcus albus urethrae*. Gram-negative and positive. First culture on ascitic agar. $\times 1200$.

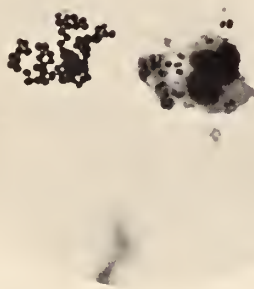


FIG. 5.—Phagocytosis of staphylococci which have been suspended in serum 3 hours. Incubation with cells 3 hours. Intracellular organisms, gram-negative; extracellular organisms, gram-positive.

The essentials for artificial production of gram-negative, biscuit-shaped, intracellular diplo-staphylococci, indistinguishable from the cocci in smears of gonorrheal pus, are the action of serum on the cocci for some hours at 37°C . before phagocytosis begins, and thereafter a period of time sufficient for complete phagocytosis to occur.

The action of antisera (staphylococcus and gonococcus) upon the staphylococci favors phagocytosis.

Suspensions in diluted serum for long periods of time act similarly to suspensions in undiluted serum for shorter periods of time.

Normal growth of staphylococci in serum and on media containing certain proportions of serum, hydrocele or ascites fluids, is characterized by biscuit forms, and there appears to be a direct relation between these forms and the gram-negative state. Single forms are rarely gram-negative.

The technic which I have used is as follows:

Methyl violet, 1 per cent.....	2 min.
Lugol's iodine	2 min.
Absolute alcohol.....	5 min.
Counterstain 1 per cent carbol-fuchsin.	

If we accept a conclusion of Benians¹ in regard to gram-positiveness of staphylococci, namely, that it is due to the action of iodine on the cell wall of the cocci, which prevents the alcohol from washing out the stain, it is possible to account for the loss of the stain by the physical change represented by the biscuit form.

It is possible, then, to produce artificially appearances identical with those of the diagnostic smear from gonorrheal pus with staphylococci, whereas with freshly isolated virulent strains of gonococci it is impossible to do so.

Of importance also in this connection is the fact that in the presence of serum and cells staphylococci elaborate anaphylatoxic substances, whereas in NaCl solution they do not.² Gonococci and meningococci, on the other hand, always gram-negative, quickly yield toxic substances in NaCl solution. Rosenow points out "the parallelism between the ease with which the various cocci disintegrate in salt solution, and the tenacity with which they retain the gram stain." There is, however, this distinction, that whereas gonococci, meningococci, and pneumococci undergo autolysis in the gram-negative state, there is no evidence to show that gram-negative staphylococci do so. It is possible that toxic substances may be liberated from them during normal growth. Gonococci behave differently in cell and serum mixtures and autolysis is hastened as compared with suspensions in NaCl solution. Freshly grown gonococci, after being in contact with cells and

¹ *Jour. Path. and Bacteriol.*, Cambridge, 1912, 17, p. 199.

² Rosenow, *Jour. Infect. Dis.*, 1912, 10, p. 118.

serum for one hour, show great diminution in numbers and marked autolysis. Heavy suspensions in weak, slightly alkaline pancreatin solutions are quite clear in 24 hours.

It is possible that the toxicity of the staphylococcus induced by serum, and possibly evidenced by the gram-negative state, exerts an influence locally in the urethra during the course of gonorrhea, and also on the body as a whole, greater than has been supposed. My experiments show that rabbits inoculated intravenously and intraperitoneally with mixed cultures of urethral staphylococci and gonococci perish from doses which, given singly, exert no ill effects. Staphylococci are recovered from the heart's blood postmortem. Abscesses frequently result from inoculations of mixed cultures, whereas staphylococci alone much less frequently cause abscess, and gonococci alone never. Staphylococci grown in broth containing gonococcus autolysate appear to be far more virulent for laboratory animals than the same strains grown in ordinary broth.

The staphylococcus in question grows well on all media, but especially on media containing leukocytic extract, and small percentages of pancreatin, which is of interest in view of the predominating trypsin-like character of the enzyme of the polymorphonuclear leukocyte and of the enzyme of the gonococcus. It is also noteworthy that media containing small amounts of alcohol are favorable to growth, particularly beer of which the acidity has been reduced. Wort and all the fluids of the beer process are excellent media. The gonococcus, on the other hand, has no predilection for such media.

Gonococcus autolysates and dried, dead gonococci produce transient irritation and purulent discharge when placed in the normal human urethra. It is of interest that sterile pancreatin, an enzyme similar to the gonococcus enzyme, produces like results. When the exudate is spread upon a slide and stained with methylene blue there are seen pus cells, some of them containing organisms indistinguishable from those observed in gonorrheal pus. Greater irritation and more copious and prolonged discharge are observed following inoculation of the normal urethra with staphylococci freshly grown from gonorrheal pus. Stained with methylene blue,

the exudate is strikingly similar to gonorrheal exudate. At an early period of the discharge the intracellular organisms are mostly gram-positive. At a late period the phagocytosed cocci are less numerous and gram-negative.

Culture experiments with phagocytosed staphylococci are easily made, whereas it is difficult to obtain cultures from phagocytic mixtures containing gonococci. The life of the gonococcus outside the body is at best precarious, and the leukoprotease of the phagocyte only serves to hasten the dissolution of the cocci, which their own similar ferment has begun. For the cultivation of the intra- and extra-cellular cocci in a manner favorable to observation of their growth there was adapted a method used by me for many years in the study of colony formation,¹ namely, that of sowing the culture mixture upon films of culture medium spread on microscopic slides, contained in moist sterile petri dishes of large size. The cultures may be examined from hour to hour under the microscope, either in the fresh state or stained *in situ* on the culture medium after fixing with methyl alcohol.

Ordinary phagocytic mixtures of staphylococci (15 strains), and of gonococci (15 strains), and of staphylococci and gonococci together, incubated at 37° C. for periods varying from 20 minutes to one hour, were employed in a large number of cultures. The medium was nutrient agar containing varying percentage of ascites fluid. The study of phagocytic preparations in this manner reveals important facts. A strong chemotaxis exists between the polymorphonuclears and staphylococci, but once phagocytosis has occurred, the cell often becomes the prey of the cocci rather than the victor over them. Numbers of the cocci multiply at the expense of the cells. The early appearance of the phagocytes, after one-half to two hours' incubation, is strikingly similar, if not identical, to that of cells in gonorrheal pus. As time goes on the cocci multiply rapidly, escape from the cell and grow first in an irregular, later in a circular manner over the surface of the medium. In the young, round, 8-12-hour colony the diplococcal, coffee-bean contours and the gram-negativeness of the individuals are well

¹ Ruediger has used a similar method in studying streptococci: *Jour. Am. Med. Assn.*, 1905, 44, p. 198.

shown, particularly at the periphery. The colonies which start from extracellular cocci do not differ save in a greater rapidity of growth. The older colonies, 24-48 hours, appear to have retained the gram stain in the mass, yet careful examination shows that this is due to the color in the cocci which are not in contact with the medium.

The cocci in colonies still so young that all the individuals are in contact with the medium are gram-negative, and the sites of old colonies that have been lifted off prior to staining show the remaining young individuals at the periphery and the old ones in the center to be gram-negative also. The spherical forms in the convexity of the colonies and the similar individuals forming colonies grown on plain nutrient agar retain the gram.

In striking contrast are the appearances of the cultures from phagocytic mixtures of gonococci. The colonies grow at one-third the rate of staphylococci, and together with the individuals composing them

present a totally different appearance. No colony is so young but that autolysis has begun. The individuals take the stain (Wright or modified Rowmanowski) poorly.

The edges of the colonies are ragged and uneven, differing greatly from the mathematical, mosaic-like regularity of the staphylococcus colonies. The colonies develop from isolated extracellular individuals and from those in contact with the cells. In no instance out of many hundred preparations examined was a colony observed to start from the few individuals which appeared to be definitely intracellular. The intracellular cocci do not resemble staphylococci or the intracellular cocci in gonorrheal pus. They remain

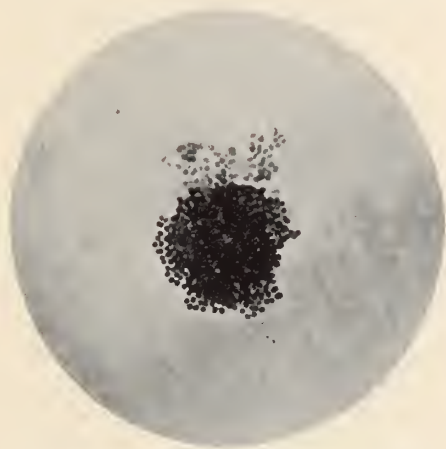


Fig. 6.—Colonies of staphylococci (below) and gonococci (above). 12 hours. From acute case of gonorrhea. Hastings. $\times 1200$.

apparently unchanged for hours and later slowly disappear. The longer phagocytic mixtures are incubated, the fewer the resulting colonies.

The contrasts are still better shown in the cultures from phagocytic preparations of mixed staphylococci and gonococci where the colonies grow side by side. The staphylococci, intra- and extracellular, grow rapidly. After 4-6 hours the former have

quite overgrown the leukocytes, while the small clusters of gonococci and the individual gonococci remain unchanged. The leukocytes themselves stain well after 24 hours.

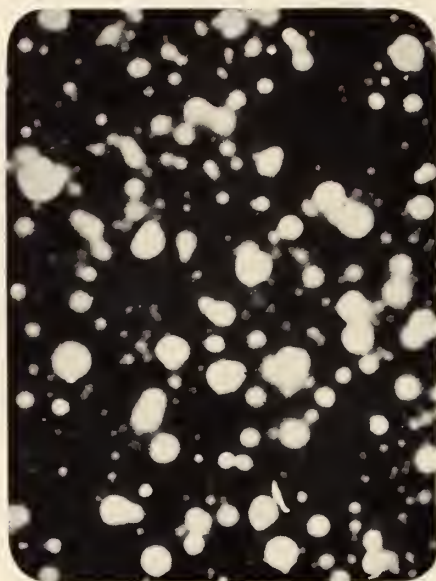


FIG. 7.—Slide-plate colonies, 24 hours old, from a case of acute gonorrhea. *Staphylococcus* colonies, large, round, dead white. *Gonococcus* colonies, small, gray, hazy and less numerous. Unstained. $\times 11$.

The same culture method was applied to the study of gonorrheal pus from 52 cases, 10 of which were observed on the occasion of each visit throughout the course of the disease. Eight or more slides were used in each culture. One or more loopfuls of pus direct from the patient were mixed in 1 c.c. of sterile ascites fluid at 37° C., and transferred at once to the culture slides

having the same temperature. Incubation proceeded at 37° .

The control smears and the culture slides fixed after one hour and stained by the same stain show all intracellular cocci to be identical in appearance. The cocci in the cultures, both intra- and extracellular, differ in no way from staphylococci and bear no resemblance whatever to gonococci. There are no visible cocci comparable to the gonococci as seen in the preceding experiments. Large numbers of the intracellular cocci appear to be dead, since they do not grow, and remain practically unchanged for a long

time. Similar appearances are noted in instances where staphylococci have remained for many hours in the phagocytic mixture before being placed on the culture medium. Many of the intracellular cocci are viable, however, and grow in a manner identical with the phagocytized staphylococci, forming characteristic colonies which on subculture yield pure growths of staphylococci.

Gonococcus colonies develop on the same plate. It is apparent that individual cocci and small groups take the stain somewhat more deeply as growth proceeds, for they are at first indistinguishable and later make their appearance by the side of leukocytes,

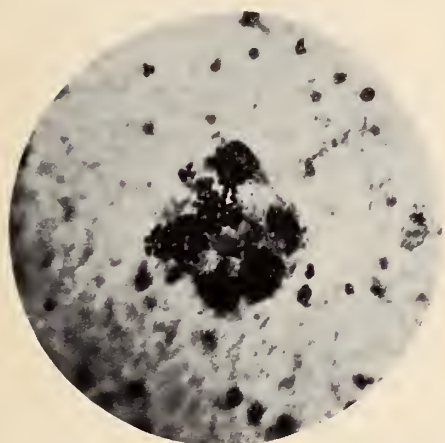


FIG. 8.—Gonorrheal pus slide-plate culture after 4 hours' incubation. Staphylococcus colony commencing in a leukocyte.

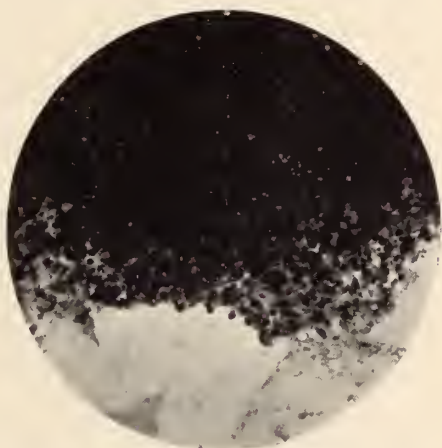


FIG. 9.—Gonococcus colony 24 hours. On ascites agar.

between them, or upon epithelial cells, and develop into characteristic colonies which yield pure cultures on transplantation. I have never observed a colony developing from unquestionably intracellular cocci.

The two types of colonies conform with those in the preceding experiments in respect to the gram stain. It is noted that individual staphylococci removed from colonies in which all the other cocci are biscuit-shaped and gram-negative often become spherical and gram-positive in smears with distilled water or salt solution.

Changes in the culture medium with respect to the ascites fluid and the salt percentages give rise to variants on both staphylo-

cocci and gonococci, but the two are never to be confused. The gonococcus grows only in a medium containing an optimum of serum or its equivalent. It is to be mistaken for no other organism except *Diplococcus intracellularis*.

Upon the same slides with these two organisms under consideration there may develop other bacteria, but the latter are easily differentiated.

It may be contended that occasionally gonorrheal pus yields pure cultures of gonococcus. It has not been my experience. Comparatively pure cultures are rare, and, in my opinion, con-

stitute a sign of impending epididymitis or arthritis. Every clinician is familiar with the diminution or actual cessation of the urethral discharge synchronous with an oncoming epididymitis. I am inclined to regard this phenomenon as due to a fresh focus (autoinoculation), not of gonococci, but of staphylococci, for the following reasons: A like phenomenon may be brought about

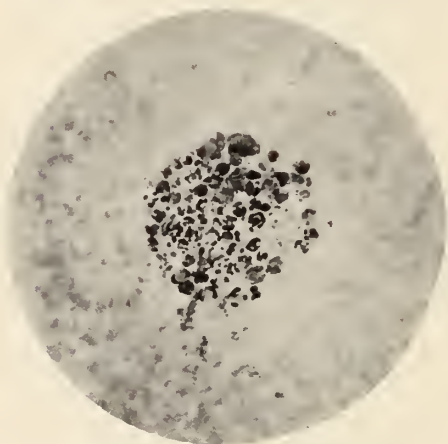


FIG. 10.—Blasted gonococcus colony showing extreme lysis.

by subcutaneous inoculations of staphylococcus suspension or vaccin. Such inoculations also cause a rapid diminution of staphylococci in the cultures, though not necessarily in smears, so that not infrequently almost pure cultures of gonococcus are obtained. On the other hand, subcutaneous inoculations with gonococcus suspensions induce no such effects. I have also obtained almost pure cultures of gonococci when the smears apparently contained no intra- or extracellular organisms whatever.

I have made a painstaking effort to identify gonococci in stained smears of gonorrheal pus, but without avail. I know of no means by which one can be absolutely certain. It has occurred to me that the rather large, poorly stained coccal forms, and the "plas-

modial" forms similar to those, often seen in gonococcus colonies where autolysis is excessive, both of which are observed, rarely within the polymorphonuclears, more often free between the cells. in smears might possibly be degenerating gonococci, but of them one cannot be sure, since they may be equally well nuclear remnants. The autolysis of gonococci is so swift under the best of conditions that the cocci stain irregularly and quickly lose their contours. How much more swiftly, then, must they vanish in the alkaline pus already laden with fatal autolysates and the enzyme of disintegrated cells?

As the infection progresses, the gonococcus comes less and less into evidence in cultures, until finally, about the sixth or eighth week, it has disappeared altogether. The time corresponds fairly accurately with that at which the complement fixation test is strongest. Of great interest in this connection is the fact that the complement fixation test for *Staphylococcus urethrae* directly parallels the gonococcus test. This test has been made in 60 cases, using as antigen a fresh suspension in salt solution of 15 strains of the staphylococcus. Under perfect control it was determined that the two tests gave like reactions up to the time when the gonococcus reaction began to disappear, whereon the staphylococcus reactions would continue positive until some time after the gonococcus reaction had become negative. It is noteworthy that in the cases of chronic prostatitis and of protracted arthritis and epididymitis which gave partial or negative gonococcus reactions, the staphylococcus reactions were still strongly positive. Controls from cases of acne and abscesses gave only partial reactions. A detailed report on this test will be made later.

I have also made cultures of the exudates from epididymes and joints, but up to the present time the cultures have yielded *staphylococcus urethrae* only.

NITRITE DESTRUCTION AS A PRESUMPTIVE TEST FOR THE DETERMINATION OF WATER POLLUTION.*

FRANK MALTANER.

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In view of the great difficulty of actually determining pathogenic organisms in waters slightly polluted, it has become necessary to formulate a number of methods, both chemical and bacteriological, the purpose of which is to determine the likelihood of pollution. The underlying principle of all of these tests, apart from the actual determination of quantities of mineral substances harmful because of their chemical nature alone, is to determine the quantitative and, to a limited extent, the qualitative bacterial contents.

The direct bacteriological methods aim at the determination of the presence of bacteria common in the intestinal tract of man and animals, and the most important of these are of course the colon bacillus tests.

The chemical methods, although indirect in their approach, have a similar aim, but attain it by determining the presence of abnormal amounts of substances excreted by man and animals which find their way into a water from a source of pollution, or the chemical results of bacterial destruction of such organic matter. Chemical examinations are of great value only when repeated tests have established the normal chemical contents of the water and when considerable variation from this normal indicates a change not attributable to such ordinary occurrences as rainfall or change in ground water levels. Such examination, however, is of most value when carried out in conjunction with bacteriological tests, the two methods correcting and checking each other. Whenever chemical investigation reveals abnormal quantities of such substances as ammonia, nitrites, nitrates or chlorids, the presumption is that they indicate pollution. Probably not being derived in the given case from mineral sources, they are present either as the result

* Received for publication March 31, 1913.

of the decomposition of organic matter by bacteria, or by the direct introduction of excreta. Since, however, under unusual conditions nitrites, ammonia, chlorids and other similar substances may increase in a given water without necessarily indicating pollution, it would be of much value were there a method available by which we could ascertain in a given case whether these substances were derived from mineral sources or were present as the result of bacterial cleavage of organic matter.

Proceeding from this premise as a point of departure, Sellards¹ and Bartow² have recently published a method of determining pollution in waters, which, if valid, would facilitate examinations of this kind. The reasoning upon which they based their investigations is as follows:

If free ammonia in any given water is actually present as the result of the growth of bacteria, these bacteria will continue the formation of free ammonia if transplanted into media especially adapted to such action. Conversely, if nitrites are entirely absent from a water, this may be due to destruction by the growth of bacteria, and such decomposition would of course be continued by these bacteria if the water is planted under suitable conditions upon media containing nitrites.

The problem to which they especially applied themselves was to determine some chemical constituent of natural waters which would serve as an indicator for the presence of bacteria from polluted sources by its formation or destruction in artificial media.

The production of ammonia was found to be of no value for this purpose since it was formed in suitable media indiscriminately, after incubation, both with polluted and non-polluted waters or with pure cultures of many bacteria whether of intestinal or other origin.

Experiments with nitrite destruction gave them better results. They found that when polluted waters were inoculated into nitrite containing broth media, and the tubes incubated at 37.5° C., a rapid nitrite destruction ensued, while in similar tubes inoculated with known pure waters, the nitrites persisted for much longer

¹ *Bull. No. 2, University of Illinois, Water Survey Series No. 7, 1909, p. 46.*

² *Bull. No. 23, University of Illinois, Water Survey Series No. 8, 1911, p. 115.*

periods. Control tests with pure cultures corroborated this in that, bacilli of the colon group invariably caused nitrite destruction within 48 hours whereas all other bacteria failed to accomplish the same result in 72 hours or longer.

They applied this "nitrite method" to the routine examination of waters in the State Water Survey, and found a reasonably accurate agreement when their method was compared with the glucose broth presumptive test for colon bacilli. The results on 155 waters gave:

72 per cent.	agreements
13	"disagreements
15	"indeterminate

The simplicity of the "nitrite method," described by Sellards and Bartow, and its apparent efficiency led us to apply it to the waters in the immediate vicinity of Leland Stanford Junior University. We prepared a nitrite broth similar to that used by them in their experiments, composed of: 6 grams of meat extract, 20 grams of peptone, 20 grams of gelatin, 0.05 grams of sodium nitrite, and 1 liter of distilled water.

The medium was adjusted to a two per cent acidity. It was then tubed in quantities of five cubic centimeters and sterilized by the fractional method.

This medium was inoculated with *B. coli communis* and *B. coli communior* and invariably showed complete loss of nitrites as soon as a fair cloudiness had appeared in the tubes, which in most cases was from four to eight hours at 37.5° C.

COLLECTION OF SAMPLES AND TECHNIC EMPLOYED.

All of the samples were collected in 8-oz., sterile, cotton plugged bottles, great care being taken in collection in order to get representative samples. In case of a flowing well the water was collected directly at the outflow, and in case of deep wells not flowing the samples were freshly pumped from the outflow of the well itself and not taken from a supply tank. The samples were taken to the laboratory and planted usually within two hours or less after collection. One cubic centimeter was planted into each of a series of nitrite broth tubes and also into fermentation tubes containing Jackson's lactose bile medium. Also in some cases fermentation tubes of dextrose broth were used, the same quantity of water being planted.

Waters were then collected from various sources including tap samples, deep well waters from unpolluted sources, and also several samples from sources supposedly polluted.

EXAMINATION OF TAP SAMPLES.

Water No. 1.—Samples were taken from the University tap supply. The source of this supply is a drilled and encased well over 200 feet in depth, well-guarded against pollution. The water showed complete nitrite destruction after 24 hours at $37.5^{\circ}\text{C}.$ in the 20 samples taken. No gas was formed on Jackson's lactose bile medium within three days at the same temperature and we were unable to isolate any colon bacilli either by this or by the dextrose broth fermentation method. In these and all other fermentation tests only 1 c.c. quantities of water were used.

Water No. 2.—These samples were taken from the Palo Alto tap supply. The source is a drilled artesian well about 250 feet in depth. Eight samples were tested and all gave complete nitrite destruction after 24 hours at $37.5^{\circ}\text{C}.$ They gave a negative gas test on Jackson's lactose bile medium and no colon bacilli were isolated.

Water No. 3.—The source of this supply is the Searsville Lake, a small mountain lake, removed from sources of animal pollution but having a large organic content of vegetable origin. Twelve samples of this water were collected and all showed complete nitrite destruction within 24 hours at $37.5^{\circ}\text{C}.$ No gas was formed on Jackson's lactose bile tubes and no colon bacilli were isolated. Several proteus-like and subtilis-like organisms were isolated which never failed to destroy nitrites after a few hours incubation at $37.5^{\circ}\text{C}.$

Water No. 4.—Samples were taken from the San Francisco Spring Valley Supply, which probably has a greater exposure to pollution than the other tap samples examined, although we were unable to isolate colon bacilli from the 1 c.c. quantities used in the experiment. This water, like all of the others, showed complete nitrite destruction after 24 hours at $37.5^{\circ}\text{C}.$ It also showed no gas production after three days growth in Jackson's lactose bile medium at the same temperature. Several proteus-like organisms were isolated and found to be the causative agents in the destruction of nitrites.

The positive results with these tap waters, which were uniformly free from colon bacilli, led us to examine the sources of the

supplies when possible and also to test several of the deep wells in this vicinity.

EXAMINATION OF DEEP WELLS.

Water No. 5.—Five samples were taken at different times during several months from the deep artesian well which supplies the town of Palo Alto. This water was inoculated into series of nitrite broth tubes, into lactose bile tubes and also into dextrose broth fermentation tubes. The nitrite broth tubes gave complete nitrite destruction within 24 hours at 37.5°C ., in every case. The lactose bile tubes did not show gas in any of the tubes after from 3-5 days at the same temperature. The dextrose broth tubes gave small amounts of gas in some instances, but careful examination of all of the tubes showing the slightest gas production failed to reveal the presence of organisms of the colon group. As in all of the experiments the nitrite broth tubes were carefully examined before the nitrite test was applied and no colon bacilli were found. A subtilis-like gelatin liquefier, and a gram-negative, non-gas-producing, non-gelatin liquefying organism, which were very active in nitrite destruction, were isolated.

Water No. 6.—The next water that was examined was taken from the deep artesian well which supplies the University. Five samples were collected and planted in series upon nitrite broth tubes, lactose bile tubes and upon dextrose broth tubes. The nitrite broth tubes showed complete nitrite destruction in every case after 24 hours at 37.5°C . The lactose bile tubes showed no gas production in from 3-5 days at the same temperature and the dextrose broth showed a small amount of gas in a few of the fermentation tubes. As before, the fermentation tubes showing the slightest amount of gas production were carefully examined and proved to be free from colon bacilli. A small gram-negative bacillus, which did not liquefy gelatin, was isolated, and gave a small percentage of gas on dextrose broth, but no gas on lactose or saccharose broth. A proteus-like organism was also isolated and both this and the preceding were very active in nitrite destruction.

Waters No. 7, 8, 9, 10, 11, and 12.—Samples were taken from six other deep artesian wells in this vicinity and were inoculated

into series of nitrite broth and lactose bile tubes. All of the nitrite broth tubes showed complete nitrite destruction within 24 hours at 37.5°C . Three-day cultures in the lactose bile medium gave no gas production in any of the tubes at the same temperature. Several small gram-negative, non-gas-forming organisms very similar to the non-gelatin liquefiers found in the Palo Alto well, were isolated, and found to destroy nitrites in the nitrite broth in less than 24 hours. No colon bacilli were found in any of these wells.

Four deep flowing artesian wells were examined, but did not show growth on the media at 37.5°C . However, they showed growth after several days at room temperature upon nitrite broth. After from one week to 10 days, when appreciable growth had formed, the nitrite test was applied and nitrites were found to be destroyed.

EXAMINATION OF POSSIBLY POLLUTED SAMPLES.

Water No. 13.—Four samples were taken from a dirty well supply tank, which was used for drinking purposes. The water was planted into series of nitrite broth tubes and also into series of lactose bile fermentation tubes. The nitrites were destroyed in 24 hours at 37.5°C . in every case. Gas was produced on the lactose bile medium and colon bacilli were isolated from the bile tubes. Examination of the nitrite broth tubes before testing for nitrites yielded several peculiar non-gelatin liquefying organisms. They were much like colon bacilli but gave unusually large gas volumes in dextrose and saccharose broth tubes after 24 hours at 37.5°C ., while only a small percentage of gas was formed on lactose broth and that not until after from 8–10 days incubation at the same temperature. All of these organisms were very active in nitrite destruction, destroying completely in a few hours when reinoculated into broth.

Water No. 14.—Samples were taken from an evidently polluted creek. They were planted as usual into series of nitrite broth and lactose bile tubes. Nitrites were destroyed within 24 hours at 37.5°C ., and gas was produced and colon bacilli were isolated from the lactose bile tubes. We were unable, however, to isolate

colon bacilli from the nitrite broth tubes, which were overgrown with proteus-like organisms. The nitrite broth was evidently more suitable for their growth than for that of colon bacilli. These proteus-like organisms were very active in destroying nitrites, from 5-16 hours being a sufficient incubation period.

Water No. 15.—Samples were taken from a flowing stream bordered with vegetation and planted into nitrite broth and lactose bile tubes in series. As in all of the other waters complete nitrite destruction took place in 24 hours or less at 37.5° C. No gas was formed on any of the lactose bile tubes during three days. Several subtilis-like organisms were isolated and found to destroy nitrites within a few hours. No colon bacilli were present in 1 c.c. quantities of the water.

In order further to test the method we experimented with intestinal and other organisms taken from the collection in the laboratory and compared these with some of the pure cultures isolated from the waters examined. We used the same nitrite broth employed in the preceding work and applied the "nitrite test" to the broth cultures. The results in Table I give the

TABLE 1.

Name or No. of Culture	Source of Culture	Time of Nitrite Destruction
B. subtilis.....	Laboratory collection	5 hrs.
B. subtilis.....	" "	17 hrs.
B. subtilis.....	" "	16 hrs.
B. proteus (a).....	" "	17 hrs.
B. proteus (b).....	" "	5 hrs.
B. proteus (c).....	" "	4½ hrs.
B. coli No. 1.....	Isolated from feces	4½ hrs.
B. coli No. 2.....	" "	4½ hrs.
B. coli No. 3.....	" "	4½ hrs.
Staph. pyog. aureus.....	From a boil	5 hrs.
Paratyphoid B.....	Loomis laboratory, N.Y.	5 hrs.
B. lactis aerogenes.....	College of P. and S., N.Y.	5 hrs.
B. enteritidis.....	Loomis laboratory, N.Y.	5 hrs.
Typhoid 65.....	Laboratory collection	16 hrs.
Proteus 2c.....	Isolated from polluted creek	4½ hrs.
B. proteus 2f.....	Isolated from San Francisco Water	5 hrs.
B. subtilis 2e.....	Isolated from Searsville water	16 hrs.
B. subtilis 2d.....	Isolated from flowing creek	5 hrs.
B. subtilis 1d.....	Isolated from flowing creek	5 hrs.
B. No. 16.....	Isolated from University well water	16 hrs.

source of the culture and the time that each culture was incubated before the test was applied. The cultures were kept under close observation, and as soon as an appreciable cloud of growth had

appeared in the tubes, the nitrite test was applied. All of the cultures listed in the table gave a complete nitrite destruction as soon as appreciable growth had appeared, which in 14 out of 20 cultures was from four to five hours. Of the laboratory strains tested only two, the *Spirillum milleri* and the *B. fecalis alkaligines* did not destroy nitrites within 24 hours at 37.5°C .

CONCLUSIONS.

The results obtained by the application of the "nitrite method"¹ of Sellards and Bartow to the natural waters in this vicinity, make it appear that:

1. Nitrite destruction is a common property of these waters, whether taken from evidently polluted sources or from the purest possible sources of supply.

2. Nitrite is not by any means a specific characteristic of colon bacilli nor of intestinal flora as a whole, but is a common characteristic of many bacteria, whether of intestinal origin or of a purely saprophytic nature.

3. Application of the nitrite destruction method to several species both of intestinal and other origin gave complete nitrite destruction in from 5-17 hours at 37.5°C ., contrary to the results obtained by Sellards and Bartow in which the same species of organisms did not show complete nitrite destruction after three days at 37.5°C .

¹ We are indebted to Professor J. P. Mitchell of the Department of Chemistry for much aid in controlling the accuracy of our nitrite determinations.

SOME CHEMICAL CONDITIONS INFLUENCING ACID-PROOFNESS AND NON-ACID-PROOFNESS IN A SAPROPHYTIC CULTURE OF *B. TUBERCULOSIS*.*

W. B. WHERRY.

(From the Laboratory of the Cincinnati Hospital, Cincinnati, Ohio.)

As has been already detailed by me,¹ a saprophytic culture of *B. tuberculosis*, when grown for a few days on such media as nutrient agar, glycerin agar, or glucose agar, was composed chiefly of bacilli which gave up the Ziehl-Nielson stain when treated with 10 per cent HCl in 95 per cent alcohol. However, in young cultures a few strongly acid-proof rods could always be found, and these increased in numbers with the age of the culture—especially in those on glycerin and glucose agar. The thick, yellow, wrinkled pellicle on the surface of +1 glycerin broth cultures grown for 4 to 6 weeks at 37° is entirely composed of acid-proof curved or straight rods imbedded in a tough matrix of unknown composition.

This extremely saprophytic culture was brought from R. Koch's laboratory, by Dr. V. C. Vaughan, in 1888. Most of the previous writers, excepting Sander, do not give the source or degree of saprophytism of their cultures. Apparently they were of a less saprophytic type and were still pathogenic. Sander's culture was of human origin and had been cultivated for about a month and a half.

While scattered observations on the occurrence of non-acid-proof bacilli in tubercle cultures have been made, it is only necessary to refer here to the observations of Tint, and Breskman² who, from a tinctorial study of old (16 years) and more recently isolated human cultures grown on glycerin agar, concluded that the acidfast property of cultures increases with the duration of growth and that non-acid-proof bacilli occur in greatest numbers in young cultures, though they may also be found in old cultures.

* Received for publication May 20, 1913.

¹ *The Lancet Clinic* (Cincinnati), 1913, 109, p. 134.

² *Jour. Am. Med. Assn.*, 1910, 54, p. 1591.

According to Chabas, Ferran¹ isolated a non-acid-proof strain of *B. tuberculosis* from an inoculated guinea-pig and was the first to show that a saprophytic strain could originate from a parasitic one.

During a study of the chemical requirements for the nutrition of my culture, notes were kept not only on the presence or absence of growth but on the morphologic and tinctorial characters of the bacilli, in the hope that a less toxic strain with some powers of immunization might result from a procedure which may be termed chemical selection.

While the studies of Sander,² Kuhne,³ Uschinsky,⁴ Proskauer and Beck,⁵ and the more recent work of Tiffeneau and Marie,⁶ Sauton,⁷ and Armand-Delille⁸ and his coworkers, have shown that the tubercle bacillus may grow luxuriantly in media of simple and known initial composition, it may be stated that the writer was unaware of most of these experiments, and hence the chemical conditions detailed below are not, in most instances, directly comparable with those found in previous work.

Further, these studies bring out points which apparently have not been noted before, e.g., some factors involved in the loss and acquisition of the acid-proof quality, and the influence of phosphates on the fermentation of carbohydrates.

The cultivations were carried on at 37° in 50 c.c. of various synthetic media placed in 500 c.c. narrow-mouthed bottles plugged with cotton. The media were sterilized in the autoclave or, when carbohydrates were present, at 100° on three successive days. When substances of a low boiling-point were to be added, this was done with a sterile pipette after the rest of the medium had been sterilized and cooled.

Some experiments, where acetates were the source of N. and C., showed that the area of exposed surface and volume of liquid apparently played no rôle in determining the rapidity of growth. Increased oxygen pressure was not used, as this is apparently unfavorable according to Moore and Williams.⁹ Unless otherwise

¹ *La salud. publ.*, 1907, 3, p. 34. References marked with asterisk were not consulted in the original.

² *Arch. f. Hyg.*, 1893, 16, p. 238.

³ *Ztschr. f. Biol.*, 1894, 30, p. 221.*

⁴ *Centralbl. f. Bakteriol.*, I Orig., 1893, 14, p. 316.

⁵ *Ztschr. f. Hyg.*, 1894, 18, p. 128.

⁶ *Compt. rend. Soc. de biol.*, 1912, 72, p. 48.*

⁷ *Compt. rend. Acad. d. sc.*, 1912, 155, p. 860.*

⁸ *Compt. rend. Soc. de biol.*, 1913, 74, p. 272.*

⁹ *Biochem. Jour.*, 1909, 4, p. 177.

mentioned the inoculations were made from +1 glycerin agar cultures.

The stock solution "A" of salts contained 0.1 per cent each of NaCl, KCl, CaCl_2 , and MgSO_4 . Solution "B" differed from the above in having 0.1 per cent Na_2CO_3 in place of NaCl. These were dissolved in redistilled water. The salts used throughout the experiments were Kahlbaum's C.P.

I. EXPERIMENTS RENDERING THE CULTURE NON-ACID-PROOF.

Transplants were made from a +1 glycerin agar culture, containing both acid-proof and non-proof bacilli, upon slants of Musgrave and Clegg's ameba agar (-1)¹ and upon slants of 2 per cent agar dissolved in distilled water (without clearing with egg white and hence without ovomucoid). Transplants upon these media were made about every two days, and one series was kept at 24-28° C. and another at 37° C. The growth always remained dryish and granular and never showed any tendency to wrinkle nor to produce pigment. On agar only the bacteria appeared as minute coccoid or diplococcoid bodies or as short rods. On the ameba agar the rods were usually longer, thinner, and often segmented or beaded. Throughout this time the rods retained the stain in Gram's method. During the first four weeks some acid-proof bacilli could always be found on careful search, but after the fifth week the growths remained non-acid-proof. When grown for 10 days under these conditions, the growth remained uniformly non-acid-proof and retained the same morphology and staining characters when grown again for three days on +1 glycerin agar. When suspended in 0.85 per cent NaCl solution, these cultures were not killed in 15 nor 30 minutes at 55° C. but were killed by heating to 60° C. for 15 minutes.

Hence it may be concluded that when kept under constant conditions unfavorable to the development of the acid-proof quality, this culture becomes wholly non-acid-proof though it still retains the stain in Gram's method.

¹ -1 = rendered 1 per cent alkaline to phenolphthalein with N/1 NaOH; +1 = rendered 1 per cent acid, by the addition of N/1 NaOH.

II. EXPERIMENTS YIELDING THE ACID-PROOF QUALITY.

A. ACQUISITION OF ACID-PROOFNESS WHEN ACETATES ARE THE SOURCE OF CARBON AND NITROGEN.

EXPERIMENT 5.

SOL. A+THE FOLLOWING SOURCES OF CARBON AND NITROGEN.

		Growth	Remarks
1	0.5 per cent ammonium acetate	+	In 22 days degree of acid-proofness like No. 2 in 12 days. Numerous minute pellicles.
2.....	Like 1. + 0.2 per cent KH_2PO_4	++	In 12 days majority rods long, thin, curved, and strongly acid-proof. 22 days, completely acid-proof; pellicles 1-4 cm. in diameter.
3.....	Like 2, + 5 per cent glycerin	-	
4.....	Like 2, + 0.2 per cent each of potassium acetate and calcium acetate	+ -	About half of rods are acid-proof.
5.....	Like 4, + 5 per cent glycerin	-	
6.....	Like 5, + 0.2 gm. MgSO_4 and 0.2 gm. NaCl	-	

A portion of the acid-proof pellicle on 2 was transplanted to similar media made up with Solution A in the one case and Solution B in the other. That inoculated onto Solution A sank to the bottom and there was no proliferation; that on B proliferated very slowly but on the 20th day, a thick, white, wrinkled pellicle covered about 4 square centimeters of surface. However, this growth was apparently derived from the non-acid-proof bacilli transplanted along with the acid-proof, for the smears made on the 20th day showed long, thin, curved, non-acid-proof rods along with a few acid-proof. This pellicle on B was again examined on the 36th day. It had developed a yellowish color and was extremely coherent. Smears showed that the matrix of the pellicle was filled with acid-proof rods and large acid-proof coccoid bodies. No non-acid-proof bacilli were present and the acidfast ones resisted prolonged decolorization.

An experiment varying the quantities of ammonium acetate and KH_2PO_4 showed the results given in Experiment 10.

On the 19th day, Cultures 2, 4, and 5, were sub-cultured on to +1 glycerin agar. In 48 hours Culture 4 yielded a luxuriant white growth, but the surface remained smooth with a ground-glass

appearance. Subcultures 2 and 5 showed no proliferation for 12 days, but on the 19th day scattered colonies were present.

EXPERIMENT 10.

		Growth	Remarks
A2.....	(Like 5A2), i.e., + 0.5 per cent ammonium acetate + 0.2 per cent KH_2PO_4	+	Reached maximum growth covering one-tenth of the surface on 8th day, white, wrinkled pellicle. Majority of rods non-acid-proof, few strongly proof. On 19th day matrix of pellicle filled with strongly acid-proof curved and straight rods.
A3.....	+ 0.5 per cent ammonium acetate + 0.5 per cent KH_2PO_4	+ —	Contaminated by mold.
A4.....	+ 0.2 per cent ammonium acetate + 0.2 per cent KH_2PO_4	+++	Reached a maximum growth on 8th day—white, wrinkled pellicle covering three-fourths surface chiefly non-acid-proof, but some strongly acid-proof rods present. On 19th day all acid-proof rods gone—coherent pellicle composed of short coccoid rods, non-acid-proof even when in masses.
A5.....	+ 0.5 per cent ammonium acetate + 0.1 per cent KH_2PO_4	++	Reached a maximum growth on 8th day—white, wrinkled pellicle covering one-fourth of surface area. Smears on 8th and 9th days like A2.

The addition of 0.2 per cent NH_4Cl to Solution B containing 0.2 per cent ammonium acetate (with or without 0.2 per cent KH_2PO_4) was unfavorable. When Solution B contained 0.2 per cent potassium acetate or calcium acetate or sodium acetate with 0.2 per cent NH_4Cl as source of nitrogen and 0.2 per cent KH_2PO_4 , growth appeared only in the sodium acetate culture and then very slowly. On the 11th day this culture showed the presence of many strongly acid-proof rods but the majority were non-acid-proof.

B. EXPERIMENTS YIELDING ACID-PROOF CULTURES, THE RESULT OF GROWTH IN THE PRESENCE OF AMMONIUM SALTS AND PROPYL ALCOHOL, AND MANNITE.

The results of growth in the presence of NH_4Cl , KH_2PO_4 , and methyl, ethyl, propyl, butyl, and amyl alcohols and mannite, have already been described¹ in detail. Briefly, the culture became strongly acid-proof only in the propyl alcohol and mannite cultures and here the rods showed one or more strongly acid-proof granules resembling the so-called "spores" of the tubercle bacillus. Many "free spores" were also present.

The following variation in the experiment was made in Experiment 12a.

¹ To appear in *Centralbl. f. Bakteriol.*, 1913.

1. +0.2 per cent NH_4Cl + 0.2 per cent KH_2PO_4 + 1 c.c. propyl alcohol. (Same as Exp. VI-13.)
2. +0.2 per cent NH_4Cl + 0.2 per cent KH_2PO_4 + 2 c.c. propyl alcohol.
3. +0.2 " " + 0.2 " " + 3 c.c. " "
4. +0.2 " " + 0.2 " " $(\text{NH}_4)_2\text{HPO}_4$ + 1 c.c. propyl alcohol.
5. +0.2 " ammon. sulfate + 0.2 per cent $(\text{NH}_4)_2\text{HPO}_4$ + 1 c.c. propyl alcohol.
6. +0.2 per cent ammon. sulfate + 0.2 per cent KH_2PO_4 + 1 c.c. propyl alcohol.

Growth was extremely slow but most rapid in Nos. 4 and 5. In about one month the entire surface was covered with small separate pellicles or with a continuous wrinkled pellicle (4 and 5). In the case of No. 3, the growth covered only about one-tenth of the surface area. Smears at this time showed that over half of the rods present were completely acid-proof throughout the series, and that in each case numerous extracellular and intracellular spore-like bodies were present.

C. EXPERIMENTS YIELDING ACID-PROOF CULTURES, THE RESULT OF GROWTH IN THE PRESENCE OF CERTAIN CARBOHYDRATES.

Again, as previously described,¹ a luxuriant growth of acid-proof bacilli, otherwise resembling the cultures in propyl alcohol and mannite, were obtained in the presence of NH_4Cl , KH_2PO_4 and levulose. With the same ammonium salt and phosphate the following carbohydrates served as sources of carbon in the following order: about equal to levulose stood glucose; only slightly less favorable, galactose. After two months the percentage of acidity to phenolphthalein varied in these cultures between 4 and 6 per cent. There was very slight proliferation where maltose, lactose, and inulin were the sources of carbon. In spite of the slight growth in these last three cultures, the reaction of the medium was changed, at least in the maltose and lactose cultures which, at the end of two months, were 6 and 5 per cent acid to phenolphthalein. Possibly the enzymes set free from the bacilli here gave rise to products unsuitable for the synthesis of fatty acids and higher alcohols.

III. INFLUENCE OF PHOSPHATES ON THE FERMENTATION OF CARBOHYDRATES.

Experiments showed that this culture could not utilize dextrose, levulose, or galactose nor the other carbohydrates mentioned above, nor the alcohol mannite, when NH_4Cl was the source of N. in the

¹ *Loc. cit.*

absence of KH_2PO_4 . This is probably to be explained on the basis of the brilliant work of Harden and Young, who were the first to show that the action of yeast zymase depended on the formation of a phosphorus-containing intermediary body—coenzyme.

Quite a series of phosphates was found to act quite as favorably as KH_2PO_4 in the fermentation of levulose but only a few were favorable to the production of acid-proof bacilli, as follows:

EXPERIMENT 14.

		Growth	Remarks
1	NH_4Cl 0.2 per cent, levulose 0.2 per cent (c.c.)	—	
2	Like 1, + 0.2 per cent KH_2PO_4	+++	Surface almost covered by pellicles in 48 hrs., when about one-half the long thin rods are strongly acid-proof. Likewise on 11th day—the acid-proof rods are often club-shaped. (Contaminated by penicillium.)
3	Like 1, + 0.2 per cent potassium phosphate (dibasic)	+++	Surface completely covered by wrinkled, white pellicle on 5th day, when the rods are shorter than in 2 and non-acid-proof. Long, thin, irregularly stained, non-acid-proof rods still present on 11th day.
4	Like 1, + 0.2 per cent sodium ammonium phosphate	+++	Like 3 on 5th day, short rods and diplococci present on 11th day.
5	Like 4, but without NH_4Cl	+++	Like 4.
6	Like 1, + 0.2 per cent sodium biphosphate (primary)	++	Long, thin, non-acid proof rods in 48 hrs. Growth covers about one-half surface. On 11th day over one-half of the rods are strongly acid-proof and show numerous extracellular and intracellular "spores."
7	Like 1, + 0.2 per cent sodium phosphate (secondary)	+++	Long, thin, non-acid-proof rods in 48 hrs. On 11th day about half of the rods are acid-proof but not strongly so. Minute, spore-like bodies present both in acid-proof and in many non-acid-proof rods.
8	Like 1, + 0.2 per cent sodium phosphate (tertiary)	+++	Like 4.
9	Like 1, + 0.2 per cent sodium phosphate (meta)	—	
10	Like 1, + 0.2 per cent calcium phosphate (primary)	—	
11	Like 1, + 0.2 per cent calcium phosphate (secondary)	++	Growth like 6 in 48 hrs., but pellicles are thicker—short non-acid-proof rods; on 11th day morphology and staining like 6. Not as many "free spores" present.
12	Like 1, + 0.2 per cent calcium phosphate (tertiary)	+++	Like 6 and 11 but the rods are smaller and the "free spores" vary greatly in size and are very numerous.
13	Like 1, + 0.2 per cent sodium pyrophosphate	+++	Surface almost covered in 72 hours. On 7th day the rods are chiefly short and non-acid-proof, but many larger and longer strongly acid-proof rods are present and many of these contain one or more strongly acid-proof granules.

It will be noted that in the above series acid-proof rods developed only in the presence of potassium dihydrogen phosphate, calcium phosphate (secondary and tertiary), sodium biphosphate (primary), sodium pyrophosphate and to a lesser degree in the presence of sodium phosphate (secondary). In the same solution (B) as above, containing 0.2 per cent each of ammonium acetate and glucose there was no growth, but luxuriant growth took place in the same mixture plus 0.2 per cent sodium pyrophosphate.

Further, a single experiment (15) showed that in Solution B containing 0.2 per cent and 0.4 per cent respectively of KH_2PO_4 and KNO_3 and 0.05 per cent levulose, there was luxuriant growth in 5 days, whereas no growth occurred in one month in a similar solution without the phosphate.

IV. EXPERIMENTS ON THE CHOICE OF VARIOUS SOURCES OF CARBON AND NITROGEN.¹

1. +0.2 per cent each of urea and KH_2PO_4 .—In three days, surface one-half covered with minute white pellicles. The thinner periphery of these pellicles was composed of minute non-acid-proof diplococcoid bodies, while the thicker central portion showed short thick rods some of which were acid-proof on the sixth day, but later, all the rods became non-acid-fast though still retaining the stain by Gram's method.

2. +0.2 per cent of KH_2PO_4 and 0.005 per cent of Theobromin.—On the 5th day a thin white pellicle covered about one-sixth of the surface and on the 19th day the surface was almost covered with thin, white, scattered pellicles composed of non-acid-proof short rods and diplococci.

A similar culture with caffein yielded no growth.

3. +0.2 per cent KH_2PO_4 +0.2 c.c. acetone (Mercks technical).—On the 5th day a hazy growth covered one-eighth of the surface; on the 19th day small scattered pellicles almost covered the surface. These were composed of short non-acid-proof rods often showing a granule at each end or three granules.

4. The following combinations yielded no growth: (a) 0.2 per cent KH_2PO_4 with the separate addition of 0.2 per cent of ammonium carbonate and ammonium valerianate; (b) 0.2 per cent of

¹ Unless otherwise mentioned, Solution B was used.

ammonium bitartrate with or without 0.2 per cent KH_2PO_4 ; (c) 0.2 per cent each of NH_4Cl and calcium lactate; (d) 0.2 per cent each of sodium formate, NH_4Cl , and KH_2PO_4 ; (e) Same as (d) but with 9 drops of formic acid in place of the formate without neutralization (reaction + 1.2).

5. +0.2 per cent NH_4Cl +0.01 per cent leucin, tyrosin, alanin, glycocoll, aspartic acid, and glutamic acid.—No proliferation in presence of tyrosin, aspartic, and glutamic acids; extremely slight in glycocoll; fair proliferation on 4th day in presence of alanin and leucin with apparent preference for alanin. On the 9th day, the alanin culture was completely covered by a thin, white, veil-like skin studded by small, yellowish masses of growth (which probably represented the inoculated fragments) and the skin extended 3-4 mm. up the sides of the bottle. The rods were long, thin, non-acid-proof, and showed a very much segmented protoplasm. In addition there were numerous coccoid bodies about one-half of which were strongly acid-proof. The findings were similar on the 24th day, but now the culture was completely non-acid-proof.

In the more delicate culture in the presence of leucin, the bacteria appeared as very minute coccoid bodies about $0.5\ \mu$ in diameter and about half of these remained acid-proof up to the 24th day. In another experiment when, along with 0.2 per cent glycocoll, various sources of N were employed in Solution A. there was fair growth in 14 days in the following order: in the presence of 0.2 per cent each of— KH_2PO_4 and $(\text{NH}_4)_2\text{SO}_4$; KH_2PO_4 and NH_4Cl ; $(\text{NH}_4)_2\text{H}_3\text{PO}_4$ and $(\text{NH}_4)_2\text{SO}_4$; and $(\text{NH}_4)_2\text{H}_3\text{PO}_4$ alone. Growth in these cultures appeared as very small thin scattered pellicles composed of non-acid-proof coccoid bodies and thin segmented rods. There was no growth in the following cultures: NH_4Cl without KH_2PO_4 , KH_2PO_4 and KNO_3 , KH_2PO_4 and KNO_2 . $(\text{NH}_4)_2\text{SO}_4$ without KH_2PO_4 .

CONCLUSIONS.

It seems worth while mentioning that when transplants were made from the cultures which had acquired acid-proofness in the presence of levulose, they showed little tendency to proliferate on +1 glycerin agar slants, but with few exceptions grew luxuri-

antly on +1 glucose agar slants. However, the resulting growths were non-acid-proof and the bacteria were of the coccoid type.

All attempts to show that the cultures in propyl alcohol and levulose—showing intracellular and free spore-like bodies—had a greater power to resist moist heat failed. All such cultures, when suspended in 0.85 per cent salt solution, were killed by heating at 60° C. for 30 minutes, though they were not killed by heating at 55° C. for 15 minutes. In a few instances, e.g., Culture Experiment XIV—6, no growth could be obtained on glycerin agar, glucose agar, nor on glycerin broth, when the subcultures were made from the unheated culture itself.

In the case of one heat test on Cultures XIV, 11 and 12, when the heated material failed to proliferate on glucose and glycerin agar, it was transferred to levulose agar slants. Still there was no growth.

However, it may be argued that the “spores” require special conditions for germination. Until the germination of these spore-like bodies has been actually seen, it may be well to consider their presence as due to especially rapid fat synthesis in one portion of the bacterial cell.

As already detailed by me, these artificially produced acid-proof and “sporulating” tubercle bacilli are readily stained in the unfixed condition by a large number of basic dyes but are not stained by Scharlach R. Yet the cultures are of a distinctly greasy and oily character when spread on a clean slide. Wells and Corper¹ have demonstrated the presence of lipase in *B. tuberculosis*, and it is known that certain bacteria, when grown in the presence of fats, will acquire acid-proof qualities. It is hardly necessary to go into detail concerning what is known of the substances giving bacteria the acid-proof quality, as the literature has been reviewed by Ritchie² and Wills.³ While only chemical analyses can settle the point, the writer believes that the results of vital staining show that in all probability there is a qualitative or quantitative difference in the fatty acid, lipid, and neutral fat content of the cultures described above and that obtaining in virulent tubercle bacilli.

¹ *Jour. Infect. Dis.*, 1912, 11, p. 388.

² *Jour. Path. and Bacteriol.*, 1905, 10, p. 334.

³ *Centralbl. f. Bakteriol.*, I Orig., 1912, 61, p. 37.

SUMMARY.

During a study of the growth of a saprophytic culture of *B. tuberculosis* in various synthetic media, it was observed that the morphology varied from minute coccoid bodies to short or long, thick or thin, straight or curved rods which were or were not acid-proof according to the conditions of growth.

The culture could be rendered non-acid-proof by continual growth under conditions unfavorable to the synthesis of fats.

The culture could synthesize fatty bodies rendering it acid-proof when such substances as acetates were the source of carbon and nitrogen; or from various ammonium salts and propyl alcohol; or from NH_4Cl and mannite; or from NH_4Cl and levulose, or from glycerin and glucose in the presence of peptone.

Various carbohydrates and the alcohol mannite were not attacked in the absence of phosphates. Various phosphates other than KH_2PO_4 favored fermentation, but acid-proof rods developed only in cultures containing NH_4Cl and levulose in the presence of potassium dihydrogen phosphate, calcium phosphate (secondary and tertiary), sodium biphosphate (primary), sodium pyrophosphate, and to a lesser degree in the presence of sodium phosphate (secondary).

Under the conditions of the experiments the sporelike bodies produced were killed by heating to 60°C . for 30 minutes. That is, they were killed unless they require some very special conditions for germination.

From the results of vital staining it seems reasonable to conclude that the chemical composition of these artificially produced acid-proof tubercle bacilli is different from that of virulent tubercle bacilli.

FURTHER STUDIES ON THE EFFECTS OF DESICCATION OF THE VIRUS OF RABIES, AND THE USE OF THIS MATERIAL IN IMMUNIZATION.*†

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Harris and Shackell,¹ using the method described by one of them,² were able to preserve for several months some of the infectivity of rabies virus by drying this *in vacuo* at the temperature of salt and ice ($-18^{\circ}\text{C}.$) Harris³ modified this by freezing the material with CO_2 snow or liquid air, and then drying the virus *in vacuo* as before. Prepared in this manner, the rabic material maintains a large percentage of its infectivity for a comparatively long time. This paper records further observations on the rate at which infectivity of desiccated virus is lost, and on the use of this material for antirabic immunization.

In the method employed, the rabic brain and cord is frozen with CO_2 snow, pulverized in a mortar and dried *in vacuo* over sulfuric acid at a temperature of -15° to $-18^{\circ}\text{C}.$ The material thus dried is sealed free from all moisture in glass tubes and kept in an ordinary ice-box ($8-12^{\circ}\text{C}.$).

For comparison of the infectivity of this material with that of cords dried by Pasteur's method, I have adopted the figures obtained by Harvey and McKendrick,⁴ who, in a large series of experiments, carefully determined the infectivity of rabic cords when dried over KOH at $23^{\circ}\text{C}.$ to be as follows: The minimal infective dose of fresh cord injected subdurally into rabbits is 0.025 mg. Of the "one day" cord the M.I.D. is twice that amount (0.05 mg.); of the "two day" cord, 8 times (0.20 mg.); of the "three day" cord, 40 times (1.0 mg.); and of the "five

* Received for publication May 24, 1913.

† Read before the meeting of the American Association of Pathologists and Bacteriologists, Washington, D.C., May 8, 1913.

¹ *Jour. Infect. Dis.*, 1911, 8, p. 47.

² Shackell, *Am. Jour. Physiol.*, 1909, 24, p. 325.

³ *Jour. Infect. Dis.*, 1912, 10, p. 369; *Ann. de l'Inst. Pasteur*, 1912, 26, p. 372.

⁴ *Theory and Practice of Anti-rabic Immunity*, Calcutta, 1907, p. 13.

day" cord, 80 times (2.0 mg.). The "nine day" cord is practically non-infective.

A curve showing this rate of loss has been plotted by these authors to demonstrate more clearly the rapid decline in infectivity during the first three days and the more gradual subsidence after the fifth day. This curve is practically identical with one representing the loss of water from day to day, and these authors conclude that "the rate of loss of infectivity of rabies cord undergoing slow desiccation is directly proportional to the rate of loss of water contained in that cord," and that "they represent two parallel effects of the same causal action." Harris and Shackell accept this interpretation, and state, by way of explanation; "It is the general belief that the attenuation of a rabic cord depends primarily upon the loss of water. Our work leads us to believe that it is the *method* of extracting the water which results in attenuation or destruction of virulence and not the extraction of water *per se*. To state it differently, slow desiccation attenuates and destroys the virus directly by reason of the concentration of salts and other substances which are in solution in the brain and cord. The action is therefore, in essence, a chemical one.

In a series of tests made upon desiccated brain and cord, I have found that 0.004 mg., when injected intracranially, within one to five days after drying will infect rabbits and produce paresis by the sixth or seventh day. Allowing for a loss in weight of 75 per cent. due to the dehydration, this quantity would be equivalent in weight to 0.016 mg. of fresh undried material. The M.I.D. is therefore about two-thirds that of fresh cord. The material, kept in sealed moisture-free tubes, loses one-half its infectivity after 21 days. In other words, the M.I.D. is then 0.008 mg. After 50 days it is 0.01 mg.; after 100 days, 0.02 mg.; after 200 days, 0.05 mg.; after 500 days, 0.1 mg.

In Chart 1 a curve is plotted upon the results of the tests. The vertical line represents the amount of infective units in a milligram of dried material; the horizontal corresponds to the number of days of desiccation (temperature 8-12° C.). The curve shows the number of infective units contained in a milligram of desiccated material at any given time.

Table 1 gives some of the details of the experiments upon which this curve is calculated. All material used in these experiments had been preserved in sealed glass tubes, and placed in an ordinary refrigerator ($8-12^{\circ}\text{C.}$). Series 190 and 193 were sealed at atmospheric pressure. The others were sealed *in vacuo*.

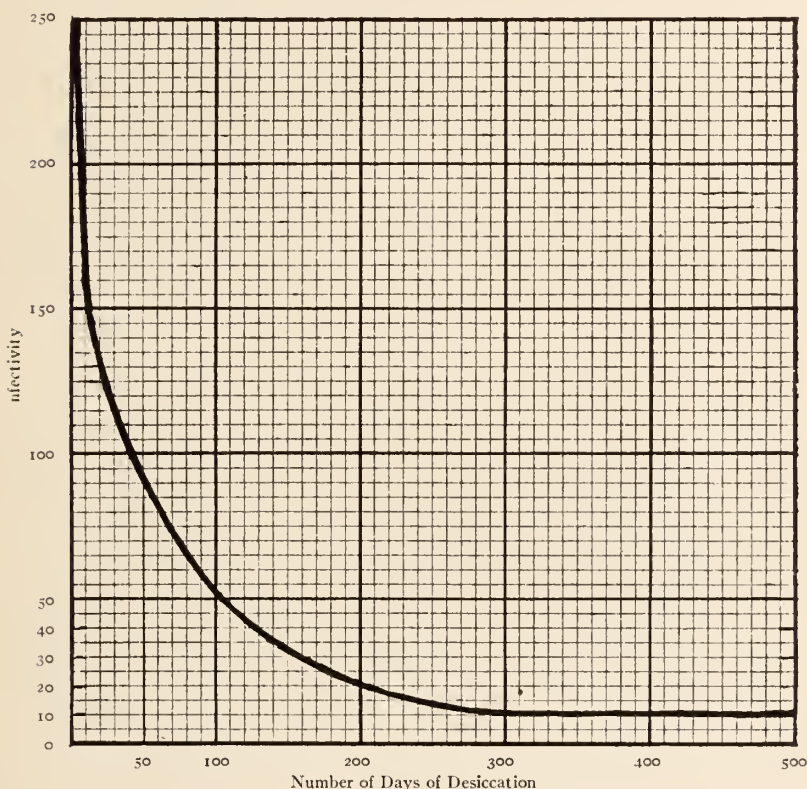


CHART 1.—Showing the rate at which infectivity of desiccated material is lost when preserved at a temperature of 8° to 12°C. The curved line indicates the number of M.I.D. in a milligram of material after the various periods of preservation shown in the base line.

In making the inoculations the usual practice is to emulsify 10 mg. of the material in salt solution which is added in small quantities from time to time up to 10 c.c. This gives a dilution of 1 mg. to each cubic centimeter of emulsion and from this other dilutions are made as desired. One-tenth ($1/10$) c.c. of the dilution is injected *into the brain* of a rabbit by trephining and passing a

TABLE 1.

Date	Series	Age of Material Days	No. of Rabbits	Amount Injected	Results
11-10-11....	184	15	6	0.02, 0.02, 0.04, 0.04, 0.2, 0.2 mg.	All paretic on the 6th day.
2-5-12....	184	97	4	(A) 0.01, (B) 0.02, (C) 0.05, (D) 0.1 mg.	Rabbits C and D paretic on 6th day. A and B survived.
3-21-12....	184	142	3	(A) 0.1, (B) 0.2, (C) 1.0 mg.	A and C paretic on 6th day. B survived.
5-8-12....	184	190	4	0.1, 0.1, 0.33, 0.5 mg.	All rabbits paretic on 6th day.
8-28-12....	184	302	2	0.1, 0.125 mg.	Both rabbits paretic on 7th day.
3-20-13....	184	502	3	0.1, 0.1, 0.15 mg.	All showed symptoms on 7th day. Paretic on 8th day.
2-22-12....	*190	2	3	(A) 0.005, (B) 0.01, (C) 0.02 mg.	Rabbits A and C ataxic on 6th day. B survived.
4-10-12....	190	50	1	0.01 mg.	Paretic on 6th day.
4-18-12....	190	58	3	(A) 0.0125, (B) 0.02, (C) 0.1 mg.	A and C ataxic on 6th day. B survived.
12-13-12....	190	205	2	0.01, 0.02 mg.	Both showed symptoms on 7th day.
3-13-12....	*193	5	5	0.0005, 0.001, 0.001, 0.002, 0.004 mg.	The rabbit injected with 0.001 paretic on 7th day. The rest survived without symptoms.
4-11-12....	193	33	4	0.005, 0.005, 0.01, 0.01 mg.	One rabbit injected with 0.01 mg. paretic on 9th day. Rest survived.
5-31-12....	193	83	3	0.02 mg. each	All paretic on 7th day.
					*This material, nos. 190 and 193, was preserved in tubes sealed at atmospheric pressure.
11-26-12....	214	31	2	0.01 mg. each	Both paretic on 7th day.
2-28-13....	214	125	2	0.02 mg. each	One paretic on 7th day. No symptoms in other.
10-26-12....	213	48	4	0.0166, 0.02, 0.025, 0.033 mg.	All four paretic on 6th day.
3-28-13....	213	201	2	0.05 mg. each	Both paretic on 6th day.
5-16-12....	202	26	3	(A) 0.01, (B) 0.02, (C) 0.02 mg.	A and C paretic on 6th and 7th day respectively. B survived.
12-26-11....	185	31	5	0.01, 0.02, 0.02, 0.05, 2.0 mg.	All five paretic on 6th day.
5-17-12....	198	27	3	0.0133, 0.02, 0.15 mg.	All 3 paretic on 6th day.
2-24-12....	187	45	2	(A) 0.01, (B) 0.02 mg.	B paretic on 6th day. A survived.
1-22-12....	185	56	3	(A) 0.005, (B) 0.01, (C) 0.02 mg.	C paretic on 6th day. A and B survived.
12-24-12....	217	1	3	0.004, 0.005, 0.005 mg.	Symptoms on 6th day. Paresis in all on 7th day.
1-15-13....	217	22	3	0.008, 0.01, 0.01 mg.	All paretic on 7th day.
1-24-13....	217	32	1	0.01 mg.	Paresis on 6th day.
2-12-13....	217	51	2	0.01, 0.0125 mg.	Both rabbits paretic on 6th day.

very fine hypodermic needle through the brain to the base, or if preferred into the lateral ventricle. With this method none of the material injected escapes after the needle is withdrawn, an occurrence which is liable to happen when the injections are made under the dura.

These tests indicate that my material, after a preservation for three weeks, with due allowance made for loss in weight, has an

infectivity equivalent to that of fresh cord; after 50 days it is 25 per cent more infective than the same quantity of the "one day" cord. After 200 days its infectivity is exactly equal to that of the "two day" cord. When kept 500 days it is two and one-half times as infective as the "three day" cord. In Chart 2 these relative values are illustrated diagrammatically.

The experiments detailed above were made upon material which had been kept in tubes *in vacuo*. A second set of tests (see Table 1, series 190 and 193) were made upon material kept in tubes sealed at atmospheric pressure. Extreme care must be taken in preparing these tubes to avoid the presence of any moisture. There does not appear to be any difference in the rate of loss whether it be preserved *in vacuo* or in dry air. When, however, there is the slightest moisture present infectivity is rapidly destroyed.

The rate of loss is greatly altered by variation of the temperature at which the material is preserved. At room temperature infectivity rarely lasts longer than five months. On the other hand, the lower the temperature, the slower the loss. One lot was kept at -1° to -3° C., and tests of its infectivity made after 32 and 75 days. Five rabbits were injected with 0.004 mg. each, and all were paretic by the seventh day. During this period there was therefore no appreciable loss in infectivity. It will be recalled that material kept for this length of time at $8-12^{\circ}$ C. loses more than three-fifths of its infectivity.

During the past 15 months 182 patients have been injected with this material for the prevention of hydrophobia. It has also been used on a large number of dogs which had been either bitten by or in contact with rabid dogs. No deaths have occurred in either the patients or the animals, and no complications have developed. Of these patients, 15 had been bitten on the face by rabid dogs, 59 on the hand, 55 on other parts of the body, while 53 had been licked on the hand or face by rabid dogs.

As this material differs considerably from the cords used in the Pasteur scheme, it became necessary, in adopting it for immunizing purposes, to follow the work of Höyges, whose method is based upon the dilution of virus rather than upon quantitative destruc-

tion in drying. Calculations show that Höyges injected in mild cases an amount equal to 54.575 mg. of fresh cord. In severe cases he gave 97.47 mg. In terms of infective units, the latter represents 3,898 M.I.D., the former, 2,025 M.I.D. According to

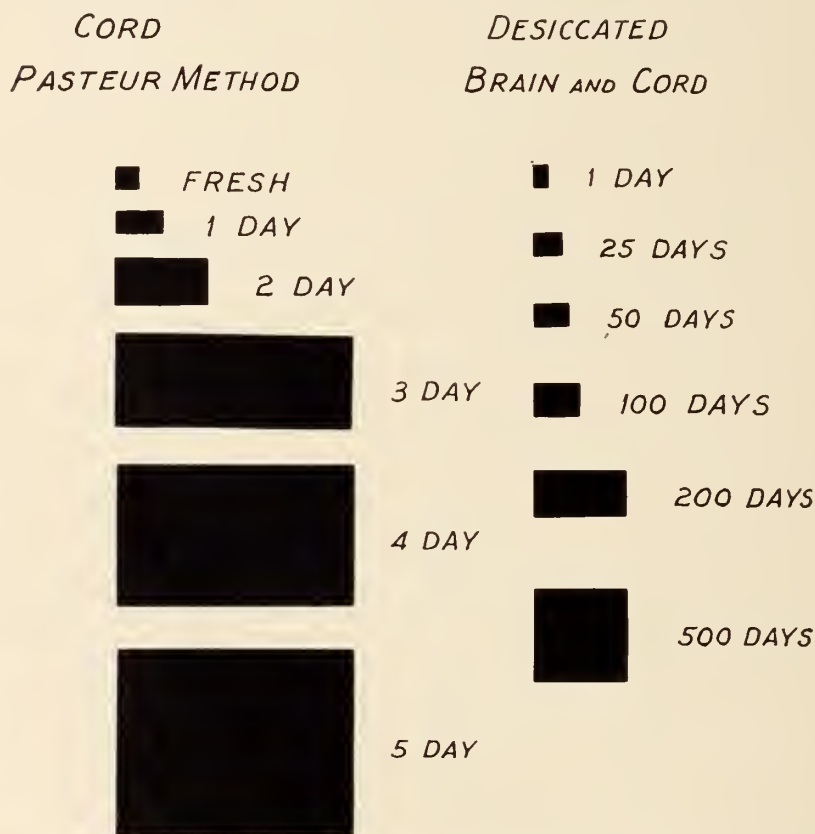


CHART 2.—Illustrating the comparative amount of cord (Pasteur) and of desiccated brain and cord which, after various periods of preservation, must be injected to produce the disease in rabbits. The M.I.D. of fresh cord is 0.025 mg. After 5 days it is eighty times this (2.0 mg.). When brain and cord are desiccated the M.I.D. is 0.033 mg. After 500 days, the M.I.D. is 0.4 mg. Thus one milligram of desiccated material 500 days old is five times as infective as one milligram of a 5-day cord.

the figures given by Harvey and McKendrick, the equivalent of the Pasteur scheme is 2,160 M.I.D.

With this as a basis, there remained the question of the quantity to be given daily and the total amount. In the light of our later knowledge concerning vaccinal immunity, I deem it unnecessary

gradually to increase the dosage to a certain point and then decrease, with alternating increase and decrease for a period of two or three weeks. I therefore have steadily increased the number of "units" (i.e., minimal infective dose) daily for the first three days and repeated this maximum quantity until a sufficient amount has been given. This total quantity depends entirely upon the location and extent of the injury, that is, upon the degree of probability of the development of hydrophobia. I also decided in mild cases to give as a minimum 5,000 M.I.D. or "units" distributed as follows: first day, 250 units; second day, 500 units; third day, 1,000 units; fourth day, 1,000 units; fifth day, 1,000 units; sixth day, 1,250 units. In ordinary cases of wounds on the hand, I give 10,000 units in 8 days, and in cases of extensive multiple, lacerated wounds of the face, I have given 30,000 units in a period of 15 days.

The question of the danger of the administration of so many infective "units" in so short a time has to be considered. In the many thousands of patients who have received anti-rabic treatment, there are very few recorded cases of infection by the fixed virus, and, though this danger is extremely remote, it has to be taken into account. In over 10,000 patients treated by the Höyges method, no such infection has been recorded. In that method, from 70 to 220 M.I.D. are injected the first day and from 200 to 400 M.I.D. the second.

Proescher and Ferran begin with comparatively large doses of fresh untreated virus. In a series of experiments Babes¹ showed that while the subcutaneous injection of fresh virus into dogs is followed occasionally by rabies, this danger can be obviated by the preliminary injection of a five-day cord. He also states that the injection of a large dose is less likely to infect animals than a small dose. The rational explanation for this apparent safety in an attenuated cord seems to be that the partially dried material contains the virus in a state so far injured as to be incapable of infecting, and yet capable of producing antibodies and a certain degree of immunity. This work of Babes led me to employ for the preliminary doses material which contains a very small amount

¹ *Traité de la rage*, Paris, 1912.

of infective units and a relatively large amount of altered or non-infective virus.

Chart 3 illustrates the relative amount of infective and non-infective material in a milligram of desiccated virus at various periods. Beginning with a maximum infectivity of one day, it will be seen that in 25 days, one-half of this has become non-infective; after 50 days, three-fifths are non-infective; after 100 days, four-fifths are non-infective; after 200 days, twenty-three-twenty-fifths. After 300 days only one-twenty-fifth of the original infectivity remains. When, therefore, one milligram of this last

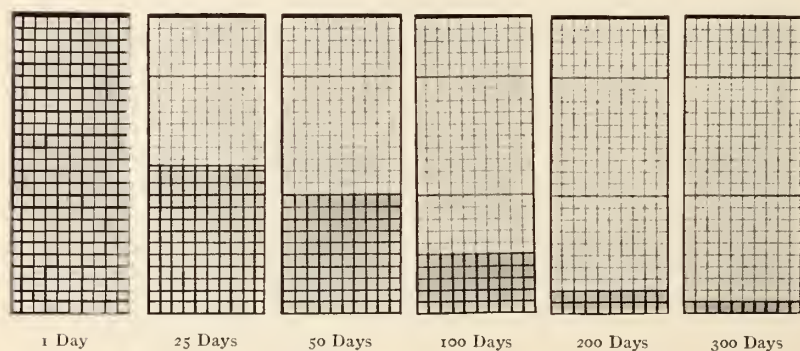


CHART 3.—Showing the relative proportion of infectious to non-infectious material in a milligram of desiccated brain and cord after preservation for various periods. The infectious portion is shaded. The number of shaded squares represents the number of "units" at any given time.

material is injected, the patient receives 10 infective units and 240 units which have become non-infective.

Several investigators have shown that after the Pasteur cords have lost all infectivity, they are but very slightly capable of producing an immunity. Harvey and McKendrick¹ state: "The immunizing power of a given portion of rabies cord is a function of the unkilld remnant of rabies virus which is contained in that cord." There is, according to these authors, no intermediate status between a "living," or immunizing, and a "dead," or nonimmunizing, state. It is well known, however, that other methods than drying may be used to destroy the infectivity of rabies virus without destroying its immunizing qualities. Babes used heat and

¹ *Loc. cit.*, p. 16.

Fermi uses carbolic acid for this purpose with excellent results. It is generally believed that, for the artificial production of active immunity, the injection of living organisms confers the greatest and most lasting immunity. In the preparation of bacterial vaccines, every agent used to kill the bacteria destroys to a certain degree the immunizing capacity of the product. Therefore, the best and safest method for the preparation of vaccines will be the one which destroys infectivity with the least degree of alteration of the antigenic substances.

Experiments seem to show that that portion of desiccated virus which becomes non-infective as time passes still possesses most of its immunizing properties. I have already shown¹ that when all the infectivity of this material has been destroyed with comparative rapidity by light and a higher temperature, it is still capable of rapidly conferring a high degree of immunity when injected subdurally. In other experiments I have conferred adequate immunity to dogs by the injection of a few doses of material which was several months old, and which contained only a very small proportion of the original infectivity.

The immunizing value of that portion of this material which has become non-infective cannot be determined with exactness until such a time has elapsed that all infectivity is lost. At present the oldest supply has been in the ice-box 500 days, and still infects rabbits in a dose of one-tenth milligram.

For the reason given above, my practice is to begin treatment with material in which the proportion of living to non-infection is 1 to 25; that is, material at least 6 months old. As the treatment continues, a gradual increase is made until material which contains 100 units per milligram is used. The fact that no accidents have occurred during the past 2 years in either patients, dogs, or rabbits is evidence of the safety and efficiency of the method.

The immunization by means of this material combines the advantages possessed by the Pasteur and the Höyges schemes. It combines the use of the attenuated and non-infectious material of the former with the simplicity of the quantitative dilution of the latter.

¹ *Jour. Infect. Dis.*, 1912, 11, p. 397.

If, as seems well established, the degree of immunity depends upon the amount of infective units injected, this method should give a greater degree of security than either the Pasteur or the Höyges scheme, since I administer from 2 to 10 times as many units to each patient as is ordinarily given.

The advantages claimed for this method of preparation are safety, economy, and convenience. It is safe because the greater portion of the material injected is capable of immunizing without being infectious. It is economical both to the patient and to the laboratory because it requires a much shorter time to administer a full treatment than most of the older methods. It is oftentimes a great hardship to require persons to leave home for a period of three weeks or more, not to mention the added cost to them for board and lodging.

It is especially economical to the laboratory in time, labor, and money. Material can be prepared two or three times a year and put aside in the cold to be used only when needed; and as one rabbit furnishes enough material to immunize from 20 to 25 patients, the initial cost of this is practically negligible. The work can be undertaken in any hospital or municipal laboratory without increasing the staff or the expense. To be able to prepare at one time material enough for from 6 to 12 months' use, and to have this always ready for any number of patients, is such a lessening of labor and anxiety as only those who have followed the classic method of drying cords can appreciate.

THE EFFECT OF QUININE ON RABIES IN DOGS.*

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The preventive treatment of rabies as devised by Pasteur is efficient only during the interval preceding the development of active symptoms, and as is well known, this treatment offers no hope after those symptoms become manifest. There is sufficient reason then why efforts should be made to find a curative remedy for rabies. It is conceded that rabies is an infectious disease, and many investigators go a step farther and class it among the diseases produced by protozoan parasites. The successful treatment of other diseases of parasitic nature, notably malaria, sleeping sickness, syphilis, etc., by internal medication should encourage efforts to discover an agent which will be effective against rabies. The fact that the preventive treatment now in use depends on the patient's system to build up its own protective substances or immunity, lends encouragement in this way—the remedy sought need not be one of sufficient strength to kill the infecting organisms outright. If a substance were found which would merely inhibit or retard the development of the infection, there is reason to believe that the system might respond by the development of antibodies as it does when injected with the attenuated virus in the course of preventive treatment. It was in pursuance of this idea that the following experiments were made.

Dogs were inoculated with rabid brain material and allowed to develop active symptoms of rabies. The technic of inoculation was as follows: a portion of the hippocampus or cerebral cortex of a rabid animal was rubbed into an emulsion with normal salt solution. With a small needle a few drops of this emulsion were then injected into and around the ulnar nerve at the elbow or a long, slender needle was inserted in the lower conjunctival sack below the eyeball and extended back until the point entered or passed through the optic foramen. The injection was thus made

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directly into the optic tract, or subdurally. The latter method of course necessitated general anesthesia, but the results were more uniform than by the other method, and the incubation period shorter. When active symptoms of rabies developed, as unusual excitability, restlessness, changed quality of the bark, decreased appetite, sometimes difficulty of swallowing, muscular spasms, paralysis of the legs, etc., the dog was given very large doses of quinine sulfate several times daily, while another dog inoculated at the same time and under the same conditions was allowed to go untreated as a control. The quinine was administered in capsules around which was stitched a thin covering of fresh tough meat as from the flank of beef. The dog would usually bolt these without chewing, but if he became unable to swallow, as happened several times, the same quantity of quinine bi-sulfate was given in solution hypodermically. The daily amount given a 6 or 7 kilogram dog was from 1.0 to 1.6 gm., usually in three doses. This is equivalent to from 12 to 18 gms. daily for an average man, in proportion to the body weight. The medication was thus pushed to the limit to secure the full physiological, bordering on toxic, effect. The idea was that in order to test thoroughly the effect of quinine, the system should be kept saturated with it. No ill effects followed, and the results seem encouraging as shown by the following data.

EXPERIMENT I.

May 14, 1912. Dogs 1 and 2 were inoculated in the optic tract with emulsion of rabid brain.

May 25. Both dogs excitable and vicious, snarling and biting when disturbed.

May 26. Symptoms continued. Quinine treatment begun on Dog 2.

June 3. Dog 1 (untreated) was vicious and savage, bark high pitched and prolonged, was getting thin, attacked food savagely, ate with difficulty, was violent and bruised the head and face against the cage.

Dog 2 was weak in the hind legs, partially paralyzed, bark hoarse and prolonged.

June 7. Dog 1 dead. Examination of the brain showed Negri bodies, large and numerous.

Dog 2 seemed nearer normal, the bark was still unnatural but the dog was not savage, nor disposed to bite.

June 9. Dog 2 apparently normal.

June 11. Quinine treatment discontinued, a total of 14.6 gms. having been given.

For a month following the treatment the dog seemed sullen and irritable of temper and would bark, growl and snap if approached. Guinea-pig inoculations to determine if the saliva were infective gave negative results.

August 1. At this time the dog's disposition seemed normal. He was perfectly docile, wagged the tail eagerly when approached and gratefully accepted petting.

August 26. Two and one-half months after the close of the treatment the dog died of unknown cause. I was unable to make an autopsy to determine the cause of death, as I was in the hospital as a typhoid patient at the time. Examination of the brain by Dr. Weber of the Chicago Health Department showed no Negri bodies.

EXPERIMENT 2.

June 6, 1912. Dogs 3 and 4 inoculated in the optic tract with emulsified rabid brain.

July 10. Dog 3 was very excitable and the bark high pitched. He continued to show signs of rabies, but the disease was allowed to run its course as a control to Dog 4. On July 23, Dog 3 died after a characteristic course. Examination of the brain showed Negri bodies.

Dog 4 showed no definite symptoms until August 3, almost six weeks after inoculation. The dog became excitable; the barks were not distinct but run together and of higher pitch than normally. Quinine treatment was begun.

August 5. Dog 4 was inattentive and disposed to lie down, was unsteady on his feet and partially paralyzed in the hind legs. Had difficulty in eating, and was unable to swallow the capsule of quinine wrapped in meat; quinine given by injection.

August 6. The dog's condition was considerably improved; he was able to eat and take the capsules as before.

August 10. Dog apparently normal.

August 12. Quinine discontinued, a total of 13 gms. having been given.

June 5, 1913. Dog 4 is alive, healthy and vigorous, apparently a normal animal.

EXPERIMENT 3.

January 14, 1913. Dogs 5 and 6 were inoculated with emulsion of rabid brain.

February 13. Dog 5 showed sudden and extremely severe onset of symptoms. Incoördination and partial paralysis first noticed in the morning. Severe tonic spasms and inability to swallow. The spasms increased in severity and length, and he died apparently of spasm of the respiratory muscles in the evening of the same day. Brain examination showed Negri bodies.

February 15. Dog 6 restless and excited, whined and barked continuously and bruised the face by striking against the cage. Quinine treatment begun at once. A guinea-pig was inoculated with saliva taken from the dog's mouth. The pig died 10 days later with typical signs of rabies and examination of the brain showed numerous Negri bodies.

February 18. The dog had marked muscular spasms, was very excitable, and unable to swallow. Quinine given hypodermically.

February 20. The dog showed partial paralysis of the legs, muscular spasms continued and the general condition was such that there seemed no hope of the dog living. The only favorable sign was that he was again able to swallow.

February 21. The general condition showed some improvement and the spasms were not so severe.

During the following days the condition improved steadily.

February 24. The dog apparently normal. Treatment discontinued, 7.8 gms. of quinine having been given.

June 5, 1913. Dog 6 is a playful, healthy, normal animal.

SUMMARY.

I have endeavored to maintain a conservative view of the results. However the following points stand out:

Of the three dogs treated two are alive and healthy at the present writing and one died of obscure cause two and one half months after treatment.

The control animals in every case died with the characteristic symptoms and pathological changes of rabies.

It is admitted by some authorities that dogs occasionally recover spontaneously from rabies. If we assume then that this was the case, and that the quinine had nothing to do with the results, we are confronted by an alternative of extreme improbability. One of the treated animals may have recovered spontaneously; that this should occur a second time is extremely improbable; that it should happen three times consecutively, and that the untreated control should die in every case is little short of impossible. However the above cases are not taken to mean that an absolute cure for developed rabies has been found. It is known that in rabies as in other diseases there are variations in severity and virulence in different cases. It is possible that these cases were of sufficiently moderate severity that the quinine furnished barely sufficient aid to enable the system to throw off the disease, and that the same result would not have followed in very severe cases. I do not assume that because quinine has been effective in dogs that it will be equally so in human cases, though this is not improbable. Viewed most conservatively these results are encouraging and indicate that the medical treatment of developed hydrophobia should not be regarded as hopeless. Should quinine not prove efficient when put to more severe tests, other agents should be given thorough trial experimentally. Upon the experimental basis already provided one would be justified in applying the same method, or rather one would not be justified in withholding the same method of treatment, from a patient suffering from hydrophobia.

In the instances above described quinine treatment was begun on the day that definite symptoms were first evident, and in human cases the quinine should be used as early as possible and freely

enough to get the full effect. The use of sedatives to quiet nervousness and excitability is in order; to prevent pharyngeal spasms on attempting to swallow, spraying the pharynx with cocaine solution has been resorted to. Curare has been used hypodermically in minute doses to relieve extreme muscular spasms in hydrophobia. Obviously all means possible should be employed to conserve the patient's strength, as exhaustion weakens the resistance to any disease. These measures are not offered as a substitute for Pasteur's preventive treatment of hydrophobia; no other treatment than that is to be considered during the incubation stage of the disease. But when active symptoms have developed, the Pasteur treatment is hopeless, while the treatment just described may offer some hope if instituted without delay.

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VARIATION IN TYPE OF INFECTIOUS DISEASE AS SHOWN BY THE HISTORY OF SMALLPOX IN THE UNITED STATES 1895-1912.*

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As many of the states have no efficient registration of deaths, and as large areas of the rural portion of the country and many cities have no notification of disease, or at least very incomplete notification, full and accurate data as to the prevalence of smallpox in the United States are not available. This disease, however, is more likely to be reported than is perhaps any other, and deaths from it, over a large part of the country, are returned with a fair degree of completeness. The table which follows is derived from the various reports and other publications of the Public Health Service. The number of deaths, except in the more sparsely settled portions of the South and West, is probably not very much too small. The number of cases on the other hand, particularly when the disease is of a mild type, is very much understated. Altho the figures for the last few years are much more nearly complete than in the early part of the period covered, at the best they indi-

* Received for publication June 16, 1913.

cate only approximately the prevalence of the disease. The figures for 1912 omit returns from a few of the states in which they are known to be very defective, as the Public Health Service does not consider it fair to other states to subject them to such comparison in its tables. Data from Porto Rico and Alaska are not included in the table.

A part of the epidemiological data has been obtained from the Public Health Service.¹ Much material has also been derived from a study of state and municipal reports, from articles in medical literature, and by correspondence. It is difficult, however, especially after the lapse of some years, and with changing officials, to obtain epidemiological data except from the best organized health departments.

CASES AND DEATHS FROM SMALLPOX IN THE UNITED STATES, 1895-1912.

Year	Cases	Deaths	Fatality Percentag
1895	2,826	589	20.84
1896	1,752	336	19.18
1897	613	38	6.20
1898	3,630	213	5.85
1899	10,957	449	4.10
1900	20,140	806	4.00
1901	48,205	1,127	2.34
1902	54,005	2,083	3.86
1903	40,675	1,448	3.56
1904	17,965	799	3.96
1905	13,046	368	2.64
1906	12,497	90	0.72
1907	17,216	59	0.34
1908	31,100	81	0.26
1909	20,072	154	0.77
1910	27,176	484	1.78
1911	23,263	105	0.45
1912	20,190	235	1.16

It must not be assumed that the general mildness of the disease is indicated by the above fatality rates. In nearly every year there were more or less localized outbreaks of the severe type, and if these be omitted, as will be done when discussing them, it will be seen that the case fatality over most of the country has been very much less than appears above.

Smallpox had been of the usual severe type and had prevailed to a considerable extent throughout the United States previous to the summer of 1897. With the exception of the mild smallpox in the South, about to be considered, and a few severe cases in the

¹ The writer is greatly indebted to Dr. Trask of the Public Health Service for much assistance.

city of New York, this disease had, by July 1, 1897, entirely disappeared from the country. In fact, during the whole year, except in the three states where it early became epidemic, there were reported to the Public Health Service less than 100 cases with about 30 deaths. These were scattered over 16 states. The local outbreaks in which these cases occurred seem to have been efficiently controlled and the disease stamped out.

During 1896 a very mild type of smallpox began to prevail in the South and later gradually spread over the country. The mortality was very low and it was usually at first mistaken for chicken pox or some new disease called "Cuban itch," "elephant itch," "Spanish measles," "Japanese measles," "bumps," "impetigo," "Porto Rico scratches," "Manila scab," "Porto Rico itch," "army itch," "African itch," "cedar itch," "Manila itch," "Bean itch," "Dhobie itch," "Filipino itch," "nigger itch," "Kangaroo itch," "Hungarian itch," "Italian itch," "bold hives," "eruptive grip," "beanpox," "waterpox," or "swinepox." Various tales were told as to its origin: that it was imported from Cuba, Mexico, or Central America, or was brought home by our soldiers after the Spanish War, but it certainly appeared before war with Spain was even declared.

From November 20, 1896, to July 10, 1897, there were in Pensacola, Fla., 54 cases of smallpox without a death and in Escambia Co., in which Pensacola is situated, many more cases also without a death. Where it came from is not known, nor whether it had any connection with the 18 cases and 2 deaths which occurred in Pensacola in May-July of that year, or with the 48 cases and 8 deaths which occurred at about the same time in Key West at the extreme southern part of the state. But as the type seems first to have been noticed in Pensacola and as there had recently been an outbreak of the usual form of the disease in that vicinity, the most likely hypothesis is that the mild type of the disease originated there as a "sport" or "mutation" from the normal, and as the normal form of the disease had, at that time, disappeared from the South, the new type had a chance to spread extensively without being mixed with the old strain.

Early in May a similar type of the disease was noticed in

Lowndes Co., Ala. It was traced thence backward to several other places and, according to Sanders, the state health officer, was derived early in the year from Pensacola. It continued to spread throughout the state and during the year ending March 31, 1898, had caused 3,638 cases with 51 deaths. It was carried from Montgomery, Ala., about the last of March, 1897, to Memphis, Tenn., and thence back to Birmingham, Ala., early in May, where it developed into an extensive and lengthy epidemic. Birmingham is a flourishing manufacturing city, with numerous rail connections, and the disease spread thence to many localities in adjoining states. It passed from Alabama to Georgia in the late summer and thence back again to northern Florida. In October, 1897, the disease was introduced from Birmingham into Middlesboro in the extreme southeast of Kentucky, whence during the next few months it spread to numerous places in the adjoining portions of Kentucky, Virginia, West Virginia, and to at least 19 different places in Tennessee.

In 1898 the new type of smallpox extended into a much larger territory and, because of its low mortality and atypical character, began to attract much attention and to give rise to all sorts of theories as to its origin. It continued in northern Florida and was imported into the center of the state from Georgia. It also continued to prevail extensively in Alabama, especially around Birmingham. Early in January, it was carried from Birmingham to Little Rock, Ark., where it caused 129 cases and 2 deaths, and gradually spread to other parts of the state. From Birmingham it was carried directly across Tennessee into many localities in Kentucky. As early as January, 1898, it had been brought to several points in South Carolina from Georgia, and at about the same time it was carried from South Carolina to North Carolina. It appeared in Norfolk, in the eastern part of Virginia, in March, but the source is not known. All through the South the larger number of cases was among Negroes who paid little attention to the disease and who, as railroad and steamboat laborers, and as servants, carried it from place to place. From Norfolk it was carried to Philadelphia late in 1898. From western Virginia, where it had been carried the year before, it spread into West

Virginia and thence to Bedford Co., Pa., in July, 1898, and thence to several other places in that state.

As early as February, 1898, it was noticed in Greene Co., Miss., adjoining Alabama and later in other parts of the state. It was also carried by a traveling show from West Virginia to New York state where there were 300 cases in 40 localities, but with only 1 death. The disease appeared at three points in Ohio, at one place brought from Georgia, and at another, a hotel, probably by colored waiters from North Carolina. In November, 1898, it appeared among boat hands in Cincinnati. From Ohio a few cases developed in Michigan, and from Michigan three outbreaks developed in Ontario. Smallpox of a very mild type also appeared in Nebraska City, Neb., as early as August, 1898, from some unknown source, perhaps, as suggested by Bracken, from Arkansas. Its nature was not discovered for some time, so that it spread rapidly, not only in Nebraska, but during the same year infected many points in Kansas and Iowa.

In December, 1898, the mild type of smallpox appeared in Soulange Co. at the western extremity of the province of Quebec. It was said to have been brought from Cleveland, Ohio. It was reported to have disappeared from this section early in 1899.

It is difficult to determine the appearance of the disease in Texas owing to the fact that in this state the problem was complicated by the presence of more or less of the severe type derived from Mexico. There appears, however, to have been mild smallpox in El Paso from August, 1898. Several colored persons were attacked and it may well be that the disease was brought by rail from Arkansas or Nebraska.

The disease must have been present in Montana in November, 1898, among railroad laborers, as a case from that source was imported into Minneapolis, December 8. According to Munn of Denver, a ranchman from Texas brought mild smallpox to Colorado in December, 1898, and distributed it through Florence, Pueblo, Boulder, Trinidad, and other places. The disease may also have existed in New Mexico in the latter half of the year, tho a very severe type was prevailing at the same time, as in La Cruces, where there were 428 cases with 166 deaths.

In 1899 the disease continued to prevail in the same mild form in all the southern states where it had occurred the year before, and also in Ohio and in Ontario. It was imported into Ontario from the states several times in 1899 and subsequent years. It was reintroduced into Michigan from Ohio and Kentucky. It spread northward from Virginia into the District of Columbia, Maryland, Pennsylvania, New Jersey, New York, and Rhode Island, tho only a few cases occurred in New England. There was a great deal of mild smallpox around Norfolk, and the many vessels plying between Norfolk and northern ports were a frequent means of dispersal. It was also again introduced into Pittsburgh, probably up the Ohio. The steamboat traffic on the great rivers seems to have been as efficient a means of dispersal as the coastwise traffic. Many places along the Mississippi River and its tributaries report infection brought by colored help on the river steamboats. As these steamboats stop at many points it is impossible, except in rare instances, to find the real source of the disease, but the states lying along these navigable rivers were, without doubt, infected from one another again and again. Cincinnati was infected from this source in 1898, and early in 1899 Newport and Covington, Ky., were infected from Cincinnati, as was also New Orleans early in February. In January Louisville was infected by a colored man from a steamboat. River towns in Indiana and Illinois were invaded early in the year from Pittsburgh, Louisville, and Arkansas, and Memphis was reinfected from the latter source. St. Louis also became infected in January and Galveston in March. Valparaiso, Ind., was infected from Mississippi in June.

Altho, as has been stated, the disease was brought to New England from southern ports, it did not seem to spread much from this source. The chief source of mild smallpox in New England for many years, beginning with 1898, appears to have been the province of Quebec. The outbreak which began in western Quebec in 1898 is said to have ceased in January, 1899, but a few cases may have lingered unrecognized. According to Pelletier of Montreal, there was no smallpox in Quebec from the last of January to August 18, at which time it was introduced into the eastern part of the province by a man from Taunton, Mass. While no smallpox

was reported from Taunton at that time, the city is close by Fall River where the disease was prevailing among the French, and French mill operatives are constantly passing from city to city. Mild smallpox has continued to prevail in Quebec ever since and has been brought by mill hands into the New England states many times and has also probably returned to Quebec from these states. Many of our New England cities are largely French and the train ride to Canada occupies only a few hours. The fact that mild smallpox appeared among French mill hands at Waterville and Winslow, Me., in January, 1899, and was present among the French in Fall River probably early in the spring, tho it was not discovered until May, suggests that the disease lingered in Quebec all winter, tho doubtless, as claimed by Pelletier, the great outbreak in August was due to a reinfection from the United States, from which also the first infection of Quebec was derived.

During the first few months of 1899 mild smallpox appeared in nearly all of the Pacific and Rocky Mountain states. According to Woods Hutchinson, at that time in Oregon, infection appeared to have come by way of Texas, California, Nevada, and southern Idaho to Oregon whence it quickly spread to Washington, Utah, and the Dakotas. According to Shoemaker of Sacramento, it was introduced into that state in February from Ogden, Utah. It was introduced into Missoula, Mont., in February from New Mexico and in March was prevailing among the Indians in Poplar and Harlem in that state. The mild type was probably present in Arizona as well as New Mexico and by June was prevailing in mining towns in Indian Territory and among Indians in Oklahoma. It was also present in Wyoming. Brought into Minnesota in January by a car conductor from the Pacific coast, it was reintroduced from Nebraska in May and later from North Dakota. It spread from Minnesota to Wisconsin.

In 1900 mild smallpox appeared among French Canadians in New Hampshire and Vermont. Early in the year it was reported from New Brunswick, being probably derived from Quebec, and in February it was in Nova Scotia. It was carried from Seattle, Wash., to Alaska and appeared in British Columbia. It was reintroduced into Colorado from all the adjoining states and was

again carried to California from Colorado, Arizona, and Kentucky. It prevailed extensively in Ontario and was reintroduced several times from Detroit and other places in Michigan. The disease became very prevalent in the middle states but there was not quite so much in the southern states where it first appeared.

From the preceding review it appears that an exceptionally mild type of smallpox had, within a period of about four years, gradually extended over the whole continent of North America north of the Mexican border. This type of the disease has been more or less prevalent ever since. The maximum prevalence was probably attained in 1902, when, outside of the communities where the severe type was prevailing, there were reported about 45,000 cases with a fatality of about 1 per cent.

The centers of greatest prevalence have of course shifted, to a considerable extent, from year to year. The disease would die out in some states, perhaps to reappear again later. This happened in several of the southern states where the disease first attracted attention. Perhaps New England and New York have had as little of the disease as any parts of the country, while the Middle West has suffered more severely and continuously. It has also prevailed continuously in the province of Quebec.

On the whole the disease seems to have shown a tendency to diminish, somewhat, in severity. This tendency is not marked and the somewhat lower case fatality noted in later years may be due to the better recognition of cases, now that the type has become more widely known. At first fatalities of 1 to 2 per cent and even more were commonly reported, while later fatalities have often been much less. Thus in North Carolina in 1910 there were 3,875 cases with 8 deaths, a fatality of 0.2 per cent, and in 1911 there were 3,294 cases in that state without a single death.

The mild type of smallpox which has prevailed so extensively in the United States was, as has been shown, also pretty well distributed over Canada and Alaska. According to Dr. Liceago it was imported into Mexico but no facts or figures are given. It does not seem to have invaded Cuba, Porto Rico, Hawaii, or the Philippines. According to Seheult¹ of Trinidad, mild smallpox

¹ *Proc. Roy. Soc. Med.*, 1908, 1, Epidem. Sec., p. 229.

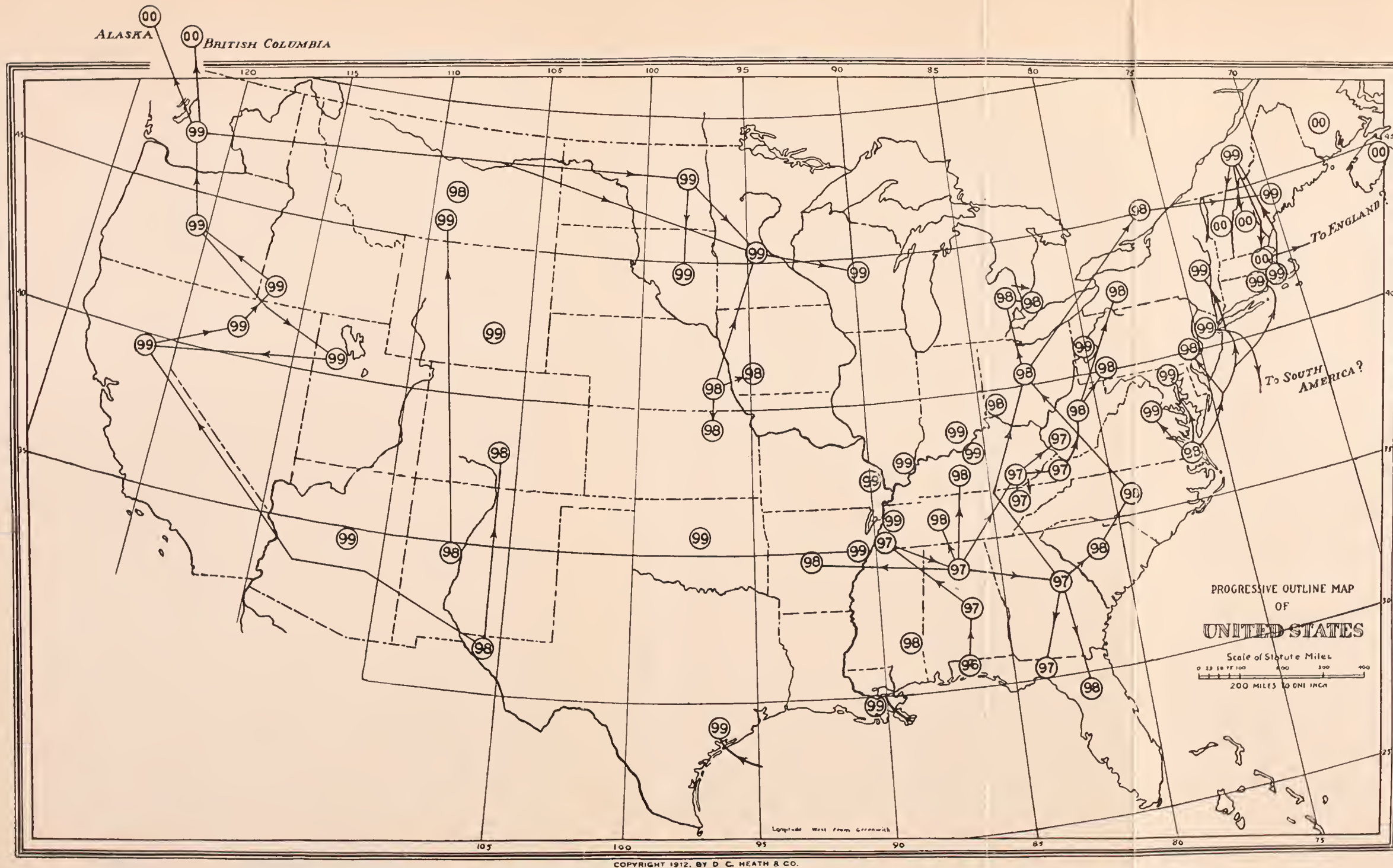


CHART 1.—Showing the extension of mild smallpox in North America, 1896-1900. The arrows indicate the course of the disease. The circles indicate the localities infected, and the figures within the circles, the year of appearance. The route of infection, when known, is shown by a line with an arrow pointing the direction, connecting the places, except that in the case of towns along the Mississippi River and its tributaries, the river itself indicates the route of infection.

was introduced into Barbados in 1902 from Venezuela where it had been prevailing for a year and where it had probably been imported from Canada. An article in the *Lancet*¹ states that it was carried from Barbados to St. Kitts, St. Vincent, Demarara, and Grenada. Carini² describes a very mild outbreak in Brazil in 1910 in which the case fatality of 5,000 cases was 2.3 per cent. In discussion Marchaux quoted Ribas as stating that the disease was introduced at Bahia.

It seems highly probable that the mild type of smallpox was carried to England from Boston in 1902 and probably during the following years. Smallpox was introduced into London, probably from Paris, in 1901 and developed into an extensive outbreak with a case fatality in that year of 14.7 per cent. The next year the fatality among 7,796 cases was about 17 per cent. In Liverpool the case fatality of 560 cases was 3.6 per cent. The disease had been introduced from Boston in December, 1901, and January, 1902, and it is probable that both types were imported as both types were certainly existent in Boston and other parts of New England. The next year there were 1,720 cases in Liverpool with a case fatality of 8.2 per cent. E. O. Stallybrass, port medical officer of Liverpool, however, writes that the outbreak in Liverpool did not impress him as being of a particularly mild type tho some such cases were discovered. It appears probably that a modicum of these mild cases was what kept down the fatality in Liverpool; and some of these, or other cases in the stage of incubation, imported from the United States in that and the following years, were the source of the mild outbreaks noted in other parts of England. Chart 1 shows graphically the extension of the mild type of the disease.

Wherever this mild type of smallpox has appeared there has usually developed a controversy as to its nature. The public and the general practitioner consider smallpox a serious disease and they are loath to believe that an affection so mild as is usually observed in this type can be real smallpox. The prodromal symptoms are not usually severe, and when the eruption appears, they disappear entirely. After that, in the great majority of cases, the patient remains practically well. Often the patient is able

¹ *Lancet*, 1903, 1, p. 1248.

² *Bull. soc. path. exot., Par.*, 1911, 4, p. 35.

to be up and about during this stage but this is not usually the case. There is usually no secondary fever and one's vocation can be followed with no discomfort. The eruption generally is not very profuse and often is limited to a few widely separated pustules scattered over the body. Quite often there may be not more than a dozen, and sometimes less. Usually the eruption passes through the same stages and presents the same general appearance as in the typical disease, but the stages are almost invariably shortened, so that from the time it first appears until the "seeds" may be picked out of the soles of the feet, even less than two weeks may be occupied, and this even when there is considerable eruption. William M. Welch says that in many of the vesicles no umbilication is seen and often on the third or fourth day the vesicles change to pustules, and almost immediately shrinking and drying begin. On many of the pustules true crusts do not appear but the exudation seems to be absorbed with little or no desquamation. Sometimes a sort of warty excrescence remains for a time but finally is absorbed. The redness left by the eruption often disappears in three or four weeks after recovery and there are, in many cases, very few or no permanent skin lesions observable. As is stated by Welch, the real cause of the difference in the eruption is its comparatively superficial character, yet even in the mildest cases the "shotty feel" is generally present and the eruption is usually at a glance distinguished from the still more superficial chicken pox eruption. The fatality is sometimes among thousands of cases as low as 0.1 per cent or even less. It is perhaps not surprising that many have been led to consider it not smallpox at all. I think, however, that health officers and clinicians all over the country, who have had an extensive experience with the old type of the disease, as well as with its present form, are convinced of their identity. The reasons are the same period of incubation, the characteristic, tho mild, prodromal symptoms in an overwhelming proportion of cases, the development of the eruption at the normal time, the distribution of the eruption, and its perfectly characteristic appearance in most cases. The crucial test of the identity of the two forms is, however, to be found in their immunity relations. It has everywhere been found that persons who have

had smallpox, or who have been successfully vaccinated, are at least as immune to the mild as to the severe type. It is also found that persons who have had the mild type are equally immune to vaccinia. Persons who have a very sparse eruption of the variola may sometimes be successfully vaccinated during the early part of the attack. It is, however, not surprising that in such a mild attack immunity is not at once established.

While atypical smallpox was spreading over the country the normal and more severe form appeared from time to time in various places causing more or less extensive outbreaks. For a number of years typical smallpox, exhibiting a considerable fatality, seemed to maintain a foothold in New York City, and in fact the abnormal mild type appears never to have extensively invaded that city. It is probable that the disease in New York was largely derived from foreign sources. Certainly many cases are each year brought on incoming steamships and occasionally one may slip by quarantine during the stage of incubation. The following table (p. 182) shows the cases, deaths, and case fatality in that city since 1898. It will be seen that an extensive outbreak of the severe type began in 1900 and extended over a period of two years.

In 1897 there were three cases with two deaths in Chicago. The disease was imported from Mexico. In December severe smallpox attacked the Zuñi Indians in New Mexico among whom it prevailed for nearly two years.

In 1898 there was considerable severe smallpox in the Southwest derived from Mexico where that type of the disease was prevailing. In December it appeared among the Moqui Indians in Arizona where in the following year it caused in a tribe of 900 persons, 590 cases and 184 deaths. There was also some smallpox in Texas at Brownsville, El Paso, and Laredo, and 30 cases with 7 deaths at Columbus. According to the Kansas State Board of Health *Report*, smallpox of the normal type was introduced by an immigrant from Russia into Marion Co., Kan., in December, 1898, where it caused 56 cases with 7 deaths. It spread to adjoining territory and also to several points along the Santa Fe Railroad. In November of 1898 there was an outbreak of 32 cases with 10 deaths in Lincoln Co., Okla., mostly in Straud and Chandler.

It extended into the Indian Territory, causing 50 deaths. There was also an outbreak of unknown origin in Carroll Co., Mo., about the same time, causing 15 deaths in 56 cases. This outbreak continued well into the spring of 1899. Perhaps the outbreak of 95 cases and 11 deaths in Kansas City during May of that year

CITY OF NEW YORK.

Year	Cases Reported	Deaths from Smallpox
1898.....	16	1
1899.....	99	18
1900.....	157	12
1901.....	1,959	410
1902.....	1,516	310
1903.....	43	5
1904.....	74	7
1905.....	46	9
1906.....	100	6
1907.....	58	9
1908.....	17	1
1909.....	9	2
1910.....	16	5
YEAR 1900		
January.....	8	1
February.....	3
March.....	10
April.....	4	1
May.....	10	1
June.....	9	1
July.....	1
August.....	2	2
September.....	1
October.....	1
November.....	39	5
December.....	70
Total.....	157	12
YEAR 1901		
January.....	70	10
February.....	128	32
March.....	220	37
April.....	200	40
May.....	450	66
June.....	304	71
July.....	244	80
August.....	118	35
September.....	27	10
October.....	23	11
November.....	47	7
December.....	62	11
Total.....	1,959	410

was an offshoot of this. There were also some severe cases in St. Louis. The location of these "normal" cases in Oklahoma, Kansas, and Missouri, all in frequent communication with the states bordering on the Mexican frontier, as well as with Mexico itself, renders it probable that this group of cases had its ultimate origin in Mexico, tho of this there is no direct evidence, except in Kansas,

where it is stated that some of the cases were derived from New Mexico.

In 1899 the severe type continued to be more or less prevalent in Arizona, New Mexico, Missouri, Kansas, Arkansas, Indian Territory, and Texas. In the latter state there were 685 cases with 126 deaths in Laredo, and 56 cases with 15 deaths in Dallas. The source was Mexico. In Los Angeles a few severe cases had been imported from New Mexico, but the disease did not spread until a fresh importation, probably from Arizona, in 1899, resulted in 93 cases with 16 deaths. There was an outbreak of the severe type in the jail at Denver in January, 1899, said to have been imported from Manila. In the latter part of 1899 there was a series of severe cases in Minneapolis, apparently derived from the mild strain. Similar series of cases occurred once or twice in that city in subsequent years and are believed by Bracken to have had a similar origin.

Altho the case fatality for the country as given in the Public Health Reports was 4.1 per cent, if the severe outbreaks in Texas, California, Kansas, Oklahoma, Missouri, and one or two other places, are deducted, there remain nearly 10,000 cases with about 150 deaths giving a case fatality of 1.5 per cent.

In 1900 so far as the United States was concerned, altho small-pox of the mild atypical type was prevailing over large areas, the typical form, with a considerable case fatality, was reported only in Louisiana and New York City. In the parishes of Caddo (Shreveport), St. Tammany, and St. Landry, La., and in New Orleans, there was, during the last few months of 1899 and the first half of 1900, a considerable number of severe cases and deaths, tho there appears to have been more or less of the mild type present also. In Shreveport there were, in January, 46 cases of which 23 were confluent, with 6 deaths. In all there were reported 19 deaths in Shreveport and 386 cases with 30 deaths in the whole parish. There were 782 cases with 27 deaths in St. Landry between September 1, 1899, and February 6, 1900, and between October 28 and December 12, 1899, there were 23 cases and 8 deaths in St. Tammany, which is across the lake from New Orleans. The other parishes are in different parts of the state. The source of the infec-

tion is not known, but, as the disease was introduced into Shreveport from Mexico earlier in 1899, tho it did not spread, it is not unlikely that it had the same source later in the year. In New Orleans there had been an outbreak of the mild type in 1899 at which time it was prevailing generally throughout Louisiana, and it was stamped out about the middle of the year. In October the disease was again introduced from an adjoining parish and from the near-by places on several occasions during the remainder of the year. In January of 1900 the Charity Hospital was infected by an imported case and the outbreak resulting therefrom caused, up to April 30, 1900, 752 cases and 252 deaths, giving a case fatality of 32.2 per cent. According to the New Orleans health report the outbreak seemed to have been checked several times but was started up afresh by importations, and more or less of the severe cases continued to appear until the end of 1901. In 1902 there were scattered through the year 34 cases with 4 deaths and in 1903, 126 cases with 4 deaths. Leaving out the Louisiana cases and deaths, the fatality for the rest of the country was 1.5 per cent.

In April, 1900, a traveler from Japan, who was infected before reaching this country, was the cause of 44 cases in Winnipeg, many of which were very virulent, with 8 deaths. From the same source severe cases also developed in Port Arthur, Carleton Place, Thunder Bay, and elsewhere in Ontario, and an infected traveler carried this type of the disease to Montreal where the mild type of the disease was prevailing, and to this source 21 cases with 8 deaths were traced between April 22 and June 15, 1900. Throughout the province of Quebec during the first six months of 1900 there were, besides the above, 692 cases with only 3 deaths.

In 1901 there appears to have been an outbreak of typical smallpox with a very considerable mortality in Memphis, occurring chiefly in the first three months of the year. There were reported 416 cases with 72 deaths, while in Tennessee, outside of Memphis, there were 1,739 cases with 22 deaths. Of these 22 deaths, 10 with 293 cases were in 6 counties, leaving 1,446 cases and 12 deaths for the rest of the state. These figures indicate that, while the mild form of the disease prevailed extensively, there was an outbreak of the more severe type in Memphis, which was perhaps also the source of some of the fatal cases occurring elsewhere.

Early in 1901 northern New Jersey was infected from New York with a virulent type of smallpox. As was previously shown, an extensive outbreak began in New York the last of December or the early part of January. The mild type had occurred more or less in New Jersey, and cases continued to appear as the result of fresh importations chiefly from the South. A peddler from New York brought the severe type to Newark in March, 1901. From that time to the end of the year there were 387 cases with 71 deaths, giving a fatality of 18.3 per cent. The outbreak continued, but with decreasing intensity, until the close of 1902, having reached its maximum in May of that year. During 1902 there were, in Newark, 901 cases with 187 deaths, giving a case fatality of 20.6 per cent. Many near-by towns of northern New Jersey, as Jersey City, Hoboken, Elizabeth, Rahway, and Orange, were infected and many fatalities occurred. The infection in many instances was traced to Newark or New York. In the whole of New Jersey there were in 1901, 1,139 cases with 142 deaths, giving a case fatality of 12.5 per cent. Besides the outbreak in the northern part of the state, there were in Gloucester, Camden, and smaller places across the river from Philadelphia many fatal cases due to infection probably derived chiefly from the latter city, tho some cases came from New York. In this portion of the state, as well as about New York, the outbreak continued well into 1902. In 1903 there was an outbreak of the normal or severe type in Bordentown and in 1904 in Camden (opposite Philadelphia where the disease was prevailing) and in Trenton.

During 1901 the normally severe type of smallpox appeared in Philadelphia and some other parts of Pennsylvania. There had been only two cases of smallpox in Philadelphia up to March, when a well marked outbreak began to develop. According to the report of the board of health it started in the northern part of the city. It spread rapidly and during the remainder of the year there were 977 cases with 186 deaths, giving a case fatality of 19 per cent. It continued to prevail during 1902 as will be noted farther on. Some cases of a severe type with a few deaths occurred in other parts of the state, particularly in near-by counties, especially in Chester and Norristown, where, in some instances at least, the disease was traced to Philadelphia.

While typically severe smallpox was extending from New York toward the West it began to appear in the East also. It happened that in May, 1901, the disease was imported into New Bedford from the Cape Verde Islands causing 37 cases with 6 deaths. There had been more or less of the mild type in Boston, and in May severe cases began to appear. Their source is not known. The Health Department at one time thought that an infected letter received by a family with sick relatives in New York was the cause, but such fomites rarely give rise to the disease. Communication with New York is frequent and rapid, and it may have been brought from that city a number of times, or it may have had an entirely different origin. A case was certainly brought from New York on a barge in May, but it was not believed to have spread from this. A confluent case from New York came to Dighton, Mass., in May, but this also was supposed to have been controlled. In Wakefield there was a confluent case imported from New York. In Providence, R.I., there were in May and June several cases of typical smallpox among Italians. The origin was unknown. An outbreak of the mild type developed in Woonsocket, R.I., in the summer of 1901, probably imported from Canada, and continued well into the succeeding year in the early part of which severe cases began to appear. Whether they were an offshoot from the mild cases, or were imported from Boston, is not known. There were in all 375 cases and 25 deaths. In Providence in 1902, besides several single severe cases from Boston, there was a group of severe cases from the same source, and another group of typical cases among Italians from an unknown source, a group of mild cases from Canada, and another mild group of unknown origin, but at the time suspected to be from the West. Most of the cases from Boston, and also those developing from them, were of the severe type. At North Mountain, Nova Scotia, there was in September, 1901, an outbreak of the severe type due to an importation from Boston. The disease was also carried from Boston to St. Johns, New Brunswick, on September 30, 1901, and gave rise to an outbreak of 102 cases with 22 deaths in that city, while in the rest of the province there were 600 cases with scarcely a death. The Boston outbreak reached its height in January, 1902, and ended in June. In all there were 1,024 cases with 190 deaths.

As was to be expected, many severe cases occurred in neighboring towns having close communication with Boston such as Cambridge, Everett, Quincy, and Malden, as well as more distant places as Worcester, Lawrence, Lowell, Fall River, and Providence. At the same time there was more or less of the mild type of the disease in southern New England including Boston. Some of this could be traced to mill operatives coming from Canada. There was such an outbreak in Northbridge, Mass., in December, 1901.

During 1902 typical smallpox continued to spread in Pennsylvania tho it is extremely difficult to obtain accurate data except from Philadelphia. In that city there were in 1902, 1,342 cases with 231 deaths, in 1903, 1,637 cases with 278 deaths. In 1904 there were 887 cases with 229 deaths, and by the end of the year the disease had entirely died out.

For the rest of the state local reports are often lacking and the state reports are unsatisfactory, but during the latter part of 1902 several localities reported the presence of the disease with a considerable fatality. Thus there were in the latter half of the year 604 cases with 108 deaths in Alleghany Co. (Pittsburgh and Alleghany), 200 cases with 24 deaths in Johnstown, 91 cases with 11 deaths in McKeesport, 92 cases with 13 deaths in Mt. Carmel, with a few severe cases and deaths in other places. It is impossible to trace the source of these outbreaks, particularly as the mild type was prevailing in many places as in Lanesboro and vicinity where there were 182 cases, without a death, derived from the state of New York. From the great amount of smallpox in Philadelphia, and the frequent communication between that city and the localities infected, it is likely that a good deal, if not all, of the severe type in the state was derived from Philadelphia, tho, as will be shown, typical smallpox prevailed in Cleveland, Ohio, after April, and it is not impossible that some of the infection, especially in the Pittsburgh region, may have come from the West. During the year 1903 typical smallpox continued to prevail in Pittsburgh, Philadelphia, and many smaller towns until well into 1904, but by the middle of the summer it had pretty well died out of the state. A curious offshoot of the Pennsylvania epidemic occurred in Crook Co., Ore., where there were 29 cases with 9 deaths caused by a farmer who had come from Pennsylvania early

in 1903. Otherwise in Oregon, smallpox, tho prevalent, was of the mild type.

There had been mild smallpox in Cleveland, Ohio, in 1901, but it had disappeared and early in April, 1902, another outbreak of the normal type developed from an Italian immigrant who was infected when he landed in Hoboken where he stayed four days before going to Cleveland. This outbreak caused 1,248 cases with 224 deaths in Cleveland in 1902, giving a case fatality of 17.9 per cent. It continued well into 1903, causing 105 cases and 22 deaths in that year.

For the next year or two outbreaks of the normal type continued to appear at various points in the Middle West but it is impossible to obtain definite data as to their origin. The location, usually in towns of considerable size, having frequent communication with Cleveland and Pittsburgh, and indeed even with Philadelphia and New York, renders it likely that the disease was usually introduced from these centers. As Cleveland was the nearest focus it is most likely to have been the source. During the first half of the year there was no severe smallpox in the state outside of Cleveland but after the outbreak became established in the latter city it appeared elsewhere. In Norwalk, Huron Co., Ohio, there were in 1902, 49 cases with 6 deaths, in Mt. Vernon, Knox Co., Ohio, 14 cases and 4 deaths, in Toledo, 119 cases and 11 deaths, and in Youngstown, 67 cases and 16 deaths. In Ohio, outside of these places and Cleveland, there were 2,792 cases with 51 deaths, giving a case fatality of 1.8 per cent. Small outbreaks with high fatality were reported from several counties in Indiana during the latter part of 1902 as Clay, Clifton, Kosciusko, and St. Joseph. Early in 1903 Columbus, Ohio, became infected and there were reported 428 cases with 59 deaths. There was a number of deaths in Cincinnati, Toledo, and Dayton as well as a few severe and fatal cases in smaller places all over the state. In Indiana during 1903, especially in the first half of the year, a considerable fatality was reported in Marion (Indianapolis) and Franklin counties. According to Dr. Hurty it first appeared in Rockport, having been brought by steamboat from Arkansas, and several other points along the Ohio River were infected from the same source. In the latter

part of the year severe smallpox began to appear among the white population in Louisville and continued into 1904. Other places in Kentucky were infected from Evansville, Ind., and St. Louis, Mo., in 1904. Chicago had had, during 1901 and 1902, a good many cases of the mild type; in 1902, 339 cases with 5 deaths, while in 1903, there were 389 cases with 47 deaths; in 1904, 358 cases with 28 deaths, and in 1905, 546 cases with 61 deaths, and since then the cases have been few and mild. It was remarked in 1903 that the type of the disease was changing, which might well have been due to the importation of typical cases from Cleveland or other points in the East where it was prevailing. In 1904 severe cases began to appear in St. Louis, which is not surprising, considering its connections with Chicago. In the year ending March 31, 1904, there had been 253 cases with 1 death in St. Louis, but in the next year there were 854 cases with 86 deaths. It continued in severe form until well into 1905, causing in that year 171 cases with 20 deaths. According to the report of the Health Department, it became mild toward the middle of the year, ceasing in August. In 1904 the severe type also appeared across the Mississippi from St. Louis in East St. Louis, Ill. It also continued in a few places in Ohio, as Cuyahoga and Montgomery counties, tho throughout the rest of that state there was a great deal of the mild type. In Indiana, especially toward the latter part of the year, and running into 1905, there was more or less of the severe form. It was especially prevalent in Terre Haute and Vigo Co. In Pulaski Co., Ark., there were reported severe cases in the autumn of 1904 in places where previously the disease had been prevailing in a mild form. Severe cases of unknown origin were reported from Charleston, S.C., early in the year and later in Florence, S.C.

During July–September, 1904, there were 63 cases with 5 deaths in North Adams, Mass. According to L. A. Jones, who made a careful study of conditions, a part of the cases were derived from Ohio, most of them from Canada, and one group from another unknown source, the latter being all mild. There were in 1904 and 1905 a few severe cases in Minneapolis but these were, according to Dr. Bracken, referred to later, derived directly from cases of the prevailing mild type.

In 1905 severe smallpox continued in St. Louis, Chicago, and in Indiana and in South Carolina. It also appeared in Michigan. In Grand Rapids there were 272 cases with 34 deaths derived from Dorr in Allegan Co. in the same state. There was also severe smallpox in Jackson, Mich., but whether it was connected with the other two outbreaks I have not been able to determine. As usual, also, there were some severe cases in New York City, due in part at least to importations. There were also some deaths in Jersey City. In the localities above referred to in 1905 there were 2,021 cases with 272 deaths, giving a case fatality of 13.4 per cent. In the rest of the country there were 11,926 cases with 96 deaths, giving a case fatality of 0.8 per cent.

In 1906, so far as can be learned from the United States Public Health Reports, there appears to have been scarcely any severe smallpox in the United States. Altho there were reported 12,503 cases there were only 90 deaths and these were widely distributed. The largest numbers of deaths reported were 6 in 38 cases in New York City, 5 in 16 cases in Pittsburgh, March to June, 6 in 126 cases in New Orleans, and 25 in 271 cases in California. Most of the deaths in the latter state were in or about San Francisco after the earthquake, and it is impossible to learn whether the apparent high fatality was due to failure to record cases, or to weakened resistance on account of privations due to the disaster, or to importations, or to change in type. Leaving out these 451 cases and 42 deaths, there were in the rest of the country 12,052 cases with 48 deaths, giving a case fatality of 0.4 per cent.

As in 1906, so in 1907, smallpox in the United States seemed to be almost universally of the mild type. There were certainly no notable outbreaks of the severe form. In all there were reported to the federal government 17,216 cases with 59 deaths. The highest fatality and greatest concentration of deaths were in New Orleans, 248 cases with 10 deaths, New York City, 134 cases with 9 deaths, and San Francisco, 67 cases with 4 deaths. Altho it is not so stated in any reports, and it has been impossible to ascertain the facts by correspondence, it is to be noted that in this, as in many other years, a number of severe and fatal cases is reported from the great seaports like those just mentioned. That many

of these cases are importations is certain. Isolated, imported severe cases were also reported from Laredo and Galveston, Tex., Spokane, Wash., and Wilmington, Del. Taking out the cases and deaths above referred to, the total for the United States was over 16,000 cases with 29 deaths giving a case fatality of 0.17 per cent.

There was no considerable outbreak of the severe type in 1908 and the deaths were even more scattered than in the preceding year. There were reported from Arizona 110 cases with 4 deaths. Most of the severe smallpox along the Mexican border seems to have been imported from the latter country and tho no evidence is forthcoming it is not improbable that some cases at least in this year may have come from the same source. In New Orleans the cases seem to have been more serious than in the rest of the country, as they were also in 1907, there being reported 220 cases with 8 deaths. In Osceola Co., Mich., there were 34 cases with 4 deaths, source untraced. In Fall River there were 8 cases with 5 deaths derived from a woman who came from England and developed the disease a few days after arrival.

Since 1908 Dr. Trask of the United States Public Health Service has endeavored to secure special reports of all severe outbreaks which he has incorporated in his annual reviews of smallpox that appear in the reports. In 1909 the mild type prevailed very extensively, being reported from 42 states. The only outbreaks of the severe form noted by Trask were at Yorkville, S.C., in March and April and in and about Norfolk, Va., in April and May. In the former outbreak there were 20 cases with 8 deaths. The source was not traced. At Norfolk there were 84 cases with 17 deaths. The disease was introduced twice, first by a mild case from Frederick, Md., from which a fatal case arose, and later by three severe cases in sailors from a warship just arrived from abroad. The other cases in the outbreak were not definitely traced to either source. There were also scattered through Texas a number of severe cases with 14 deaths. No source is given for these, but the statement has been made by an experienced health officer in that state that, while mild smallpox has been epidemic for some time, practically all of their severe cases are due to importations, usually

from Mexico. If the deaths at Yorkville, Norfolk, and Texas are omitted, the case fatality for the rest of the country would be only about 0.5 per cent.

The number of cases in 1910 was greater than in the preceding year and the number of deaths more than double. Nevertheless over most of the country the disease continued to present a very mild type. Thus in North Carolina there were 3,875 cases with only 8 deaths. There were, however, a number of outbreaks of the severe type. Thus there were three such outbreaks in Michigan. The mild type had previously been prevailing in the localities in question and in March severe cases began suddenly to appear in Bay City, continuing until October; then the disease was carried to Saginaw. In Bay City and neighborhood there were 134 cases with 31 deaths and in Saginaw and vicinity 239 cases with 55 deaths. Shortly afterward the disease appeared in Lapeer, which is not far distant on a through rail connection. In that city, there were 36 cases with 17 deaths. The first case was an inmate in an institution who received visitors from Saginaw.

In April smallpox was brought to Cleveland, Ohio, by a foreign case imported through Philadelphia. There were 62 cases with 10 deaths.

While there was much mild smallpox in Texas there were several outbreaks of the severe type in Bee and Denton counties during the early part of the year. The source of the Bee Co. outbreak was Mexico, that of Denton Co. was not reported. So too in Oklahoma, tho in general the disease was mild and prevalent, there were outbreaks of the severe type in Grady and Oklahoma counties from March until late in the year. In Reno Co., Kan., the mild type prevailed during the year, but in July there were 3 deaths out of the 8 cases reported. While none of these outbreaks was traced, the relation in time and space renders it not improbable that they may have had their origin in Mexico. In Bellingham, Wash., there was a small outbreak of the severe type, 5 cases with 2 deaths, traced to Vancouver, B.C., and presumed to be contracted from some foreigners recently arrived at that port. In Marion Co., Ore., there were 17 cases with 9 deaths due to exposure to a case on a train running from Mexico City into the

states. In Philadelphia there were 10 cases and 2 deaths, all cases from foreign sources.

The 672 cases included in the above mentioned severe outbreaks yielded 180 deaths, giving a case fatality of 26.7 per cent, while among the other 26,504 cases in the country there were only 304 deaths, giving a case fatality of 1.1 per cent.

In 1911 there were reported 97 cases with 25 deaths in El Paso Co., Tex., and 5 cases with 2 deaths in Guadalupe Co. The former was not traced, but the latter was from Mexico and the health officer stated that in his opinion all the severe smallpox in the state during the last 10 years was from that source. In November the malignant type of the disease appeared in Los Angeles. There had been some mild smallpox in the city during most of the year, but with no fatalities. In November, however, the virulent type appeared in the quarter of the city occupied by Mexicans, and, according to the health officer, was probably introduced from Mexico. From this time until the end of February there were 71 cases with 8 deaths. There were then 32 cases without a death, until July, 1912, when virulent cases again appeared, also from Mexico. This seems to be another instance in which the severe type was imported into a locality where the mild form was prevailing. In Topeka, Kan., there were 143 cases between May and October, with 23 deaths, apparently derived from a mild case of unknown origin. Omitting these severe cases, the case fatality for the rest of the country for the year was 0.2 per cent.

Besides the severe smallpox in Los Angeles in 1912 derived from Mexico, there were, in July, 3 cases in Pasadena with 2 deaths, the source being Los Angeles. In and about Pittsburgh there were, during the summer and autumn of 1912, 121 cases with 33 deaths. The first case was an importation from abroad, and tho connection with it was not demonstrated, the subsequent cases were among foreign residents in the same part of the city. As usual there was considerable smallpox of the severe type in Texas. In Tarrant Co., where Fort Worth is situated, from January 19 to May 22, there were 163 cases with 46 deaths. There were said to be two foci, one derived from Mexico and the other from Oklahoma. The disease was carried from this point to McCullough

Co., causing 10 cases with 4 deaths, and also to Dallas and thence to Hunt Co. Deducting the 853 cases with 168 deaths which occurred in Texas, Pittsburgh, and Los Angeles, there remain for the rest of the United States 19,334 cases with 67 deaths, giving a case fatality of 0.35 per cent.

Various theories have been advanced concerning the source and persistence of mild smallpox in the United States, most of which are clearly shown by the facts to be untenable. It cannot be due to any climatic or other environmental conditions, or to racial characteristics, for it has overspread nearly the whole continent and has appeared in the West Indies, South America, and South Africa and perhaps in England. It has affected all nationalities, and Negroes and Indians as well as whites. It has been urged by many that the mildness of the disease has been due to an acquired immunity due to the vaccination of many generations, but it has prevailed extensively in the United States, the least vaccinated of any civilized country, and reliable observers have noted its occurrence in persons whose parents were never vaccinated. Moreover the classical type of the disease whenever introduced does spread as readily as ever it did, and over large areas.

The mild type of smallpox was certainly not brought back by our soldiers from Cuba, Porto Rico, or the Philippines, for it appeared before the Spanish War was declared and the type has not occurred in those islands.

The disease appeared first in our southern states, probably in Florida in 1896. Its extension from that focus can be definitely traced over the larger part of the United States, Canada, and Alaska. It is true enough that its importation cannot be determined for a number of places, but the location and date of infection of these unconnected foci are such as to render it in the highest degree probable that they too were infected from the original source. Notable among such unconnected foci may be mentioned Nebraska City, Neb., Norfolk, Va., and Fall River, Mass. As there had been previously no smallpox of any kind in these places it can scarcely be assumed that the mild form was there derived from the normal type.

It appears, then, that the prevailing mild type was in all

probability a variation or mutation of the more usual type of the disease and that the entire North American outbreak was derived from the 1896 focus in Florida. It is of course possible that similar mutations may have occurred elsewhere in the United States but the history of this great epidemic, as given in the preceding pages, renders this supposition rather improbable. Everything indicates that the type arose suddenly, spread from a single focus, and has maintained itself with great persistence. As three weeks are perhaps more than the average time required for one case to develop from another, the virus of some of the cases existing today has probably passed through scores and perhaps hundreds of persons.

That, during this epidemic, severe cases have sometimes developed from mild ones is certain. Confluent cases and even deaths have occurred among large series of mild cases, tho perhaps it has less commonly happened in the last few years than formerly. That fatalities are sometimes due to weakness or intercurrent diseases is probably true. That the severity of the case is sometimes due to a special susceptibility to the virus is not improbable. That these severe cases which occasionally develop from the mild strain are due, sometimes, to a heightened virulence of the infecting organism is also probable. This is indicated by the fact that several severe cases in unrelated persons may develop from a mild strain. Bracken has carefully reported several such series as occurring in Minnesota in 1899 and Wood reports a similar series from the same state in 1912. Other instances have been noted in Hartford, Conn., and North Adams, Mass., and doubtless in many other places, but these will serve for illustration. A perusal of health reports and accounts of outbreaks suggests that during the last 15 years there must have been among the hundreds of thousands of mild cases, several hundred, and more likely several thousand, severe cases derived from them. Yet there have been certainly very few real outbreaks from such a source. This is not because severe cases are always recognized and efficiently isolated, for we have seen that when severe cases are imported the disease does spread. There may have been some outbreaks of the severe type derived from the mild type, but there cannot have been many, and it may be that there have been none. The mild type tends

to persist. The few severe cases derived from this type do not tend to persist. They are apparently not mutations but mere variations which tend to revert. The mild type, however, seems to be a true mutation which has shown a strong tendency to maintain its characters for at least 15 years.

The classical type of the disease, whenever it has been imported, has also shown its customary tendency to "breed true" and maintain its well known group of symptoms. The outbreaks are stamped out or run out and do not degenerate into the mild type.

The evidence points to the existence in North America during the last 15 years of two quite distinct strains of smallpox, one the long recognized type of the textbooks, the other marked by decided mildness of symptoms. The latter is probably a mutation from the former. Both strains tend to breed true, and tho it is possible that a few outbreaks of the severe type may have developed from the mild type there is no conclusive evidence that they have been numerous, or extensive.

EQUINE PIROPLASMOSIS IN PANAMA.*

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(WITH PLATE 3.)

The writer discovered recently at Ancon a fatal case of piroplasmosis in an American driving horse. According to Professor Nuttall,¹ who has made an exhaustive study of the geographical distribution of piroplasma, this is the first record of the parasite (*Piroplasma caballi*) in America. The disease, analogous to Texas cattle fever, is very common in parts of South Africa, where it is called biliary fever. According to Bowhill² it was first observed in 1883, by Wiltshire in Natal, who named the malady anthrax fever. Hutcheon, in Cape Colony, described it as biliary fever of the horse. The disease has also been reported from Italy (Guglielmi, 1899), Russia, India, China, and Brazil.

Case history.—The horse was a driving horse (No. 35), one of the first to be sent from the United States to the Canal Zone for the Commission in 1904-5.

On January 7, 1913, he was one of a pair used to carry a party of engineers on an inspection trip to Old Panama and the Juan Diaz River. The trip was made during one day, and at the river the animal was permitted to graze on the Sabannas, where there were some native ponies. Previous to the trip he had been constantly used at Ancon and the city of Panama. The animal continued at work every day until January 11, when he was taken out of the break with symptoms of overheating; temperature was 105-106.2°. The following day the temperature ranged between 102° and 105°. Respirations were labored and the pulse was feeble. On Monday, January 13, the lungs were clear, respirations were rapid, and the pulse very weak. The temperature was 105.2° at 9 A.M., and 106.2° at noon. On January 14, the animal was down, but was able to get up. Respirations were much labored and the conjunctivae were deep yellow in color with large, rather thin ecchymoses; death occurred at 12 noon.

The autopsy was performed at 3:30 P.M. Several ticks were taken from the animal's ear and flank. The animal appeared to have lost considerable weight within the last 24 hours. There was no subcutaneous edema and the cavities were clear. The right lung was congested and edematous; left normal. The right bronchus contained jaundiced mucus. In the left ventricle of the heart there were large, dark red, sub-endocardial ecchymoses. The liver was not appreciably enlarged. On section, its parenchyma was coarse, each lobule slightly separated from its neighbor,

* Received for publication June 27, 1913.

¹ Personal communication to writer.

² *Jour. Hygiene*, 1905, 5, p. 7.

and light grayish brown in color. Near the anterior margin and on the superior surface were large purplish areas of necrosis in which the central and intermediate zones had the hyaline yellow appearance of advanced fatty change. These areas in size varied up to 3 by 1.5 by 1.5 cm., and seemed to surround certain peripheral branches of the hepatic veins. None were seen in the interior. The bile ducts were normal, and contained bile, mucous in character, full of fine black dots.

The kidneys presented nothing of note. The mucosa of the pelvis in each one was covered with jaundiced mucus containing fine black dots like those noted in the bile ducts.

The urinary bladder contained turbid jaundiced urine.

The lymph nodes draining the kidney were not enlarged, or but very slightly so, yet they were intensely red in color.

The adrenals appeared normal.

The spleen was enormously enlarged. Its capsule was smooth, and of a bluish steel color free from ecchymoses. On section, the pulp bulged above the capsule and was very dark in color. Upon scraping, the pulp was removed with ease, leaving a clean gray reticulum showing but the slightest evidences of lymphoid tissue. The malpighian bodies were atrophied. The weight of the spleen was estimated as 28 pounds, and it appeared to be larger than the liver.

The great size of the spleen was the most striking feature of the autopsy, and it is readily seen how Wiltshire named the disease anthrax fever, for the size and consistence of the organ were exactly like those of anthrax of cattle.

Circumstances point strongly to the infection having been contracted from infected ticks obtained at the Juan Diaz River, for this disease has not been detected among the corral horses during the period of the American occupation, altho since 1909 careful blood examinations have been made on many of the animals, and autopsies held on practically all fatal cases of disease.

It is in such a location as Juan Diaz that a corral horse could come into contact with infected ticks from native horses, and we know that piroplasmosis is a tick-transmitted disease, one form in Russia being transmitted by *Dermacentor reticularis* and another in South Africa by *Rhipicephalus evertsi*. Ticks were removed from the horse by hostlers a day or two before the animal fell sick, and at autopsy ticks were collected which were very kindly identified for me by Professor Nuttall and Mr. Warburton as *Dermacentor nitens* and *Amblyomma cajennense*.

The parasite.—There was no gross peculiarity of color noticed in the animal's blood, yet it was hyperfluid, and thin films for staining were made with difficulty. This turned out to be no great

disadvantage in diagnosis, for the non-infected erythrocytes were clumped, leaving the few infected ones free in clear spaces where they could be detected easily. There appeared to be some degree of leukocytosis with a predominance of transitional and mononuclear forms. Eosinophils were not encountered, tho present in good numbers in two non-infected controls. The types of parasites encountered were those described by Nuttall and Strickland¹ as *Piroplasma* (*Babesia*) *caballi*, and, as these observers have noted in their experimentally infected animals, parasites were sparse.

It was seen at once that the parasite belonged to the type *Piroplasma* and not to *Nuttallia* (França 1909). The predominating forms were somewhat oviform. But a number of double pyriform forms were seen also, and a few single pyriform forms as well. Nearly all the forms illustrated by Nuttall and Strickland were found, as well as some dwarfed or attenuated forms (Fig. 10).

Koch, in 1905, stated that he believed that there were two types of parasites in equine piroplasmosis and that they might cause distinct diseases. In 1910, Nuttall and Strickland published the results of their investigations which showed that two distinct species of piroplasma occurred in horses suffering from biliary fever, and later, in 1912, they published a detailed account of their work from which it appears that the disease is caused by two parasites: *Nuttallia equi* Laveran and *Piroplasma* (*Babesia*) *caballi*, Nuttall, 1910.

According to Nuttall and Strickland, França (1909), convinced of the difference between piroplasma and Laveran's parasite, which presented "cross forms," placed the latter parasite in a new genus named *Nuttallia*. The parasites belonging to this genus do not multiply according to the method described for piroplasma. They do not occur as pairs or multiples of pairs of pyriform parasites inside of corpuscles, and they form distinctive "cross forms" which Bowhill and França regard as multiplication forms.

Period of incubation.—Infection by direct inoculation appears to be accomplished with difficulty. Nuttall and Strickland, however, noted the appearance of *Piroplasma caballi* in the blood of experimentally inoculated horses after 8, 10, and 15 days, respec-

¹ *Parasitology*, 1912, 5, p. 65.

tively. In the case reported here, one of natural infection, the period of incubation, assuming as we must that the infection was contracted at Juan Diaz, was four days.

Symptoms.—The symptoms are those of an acute infection, with sudden onset and high fever. The respirations are accelerated and the pulse is weak. Icterus of the visible mucosae is well marked, and the ecchymoses on the conjunctiva would seem to be a common characteristic. Very similar hemorrhages, however, are seen in murrina, the trypanosomal disease of horses of this region. The urine is jaundiced and highly colored, and hemoglobinuria is said to occur in some cases. The duration of the disease would seem to be variable. Some acute cases last only two or three days, in other instances much longer.

The native horses in a region would seem to be more or less immune to the disease. Bowhill's experience led him to believe that this immunity depended upon the animal being reared in an infected area. Imported animals kept separate from native stock, as is usually the case here with Commission animals, may never contract the disease. However, if they are permitted to run in native tick-infested pastures, or on the highly infested trails, it is likely that, as in the case reported here, the disease may present itself and assume serious features. Carini¹ states that among imported horses the disease becomes truly epidemic.

Theiler, according to Bowhill, states that a bipolar-staining bacillus frequently appears as a terminal or associated infecting agent. This is interesting and seems to be analogous to the associated infections by the typhoid-colon group *B. icteroides*, *B. paracolon*, and *B. suipestifer* seen in yellow fever, *Verruga perusna*, and hog cholera.

All the piroplasma exhibit a high specificity for their respective hosts, and *P. caballi* appears to be no exception to this rule.

Transmission.—In Russia, *P. caballi* is transmitted by *Dermacentor reticularius*. It seems likely then that *D. nitens* may be the carrier for *P. caballi* in this region. This tick is known as the tropical horse tick and is distributed throughout Mexico (?), Central America, the West Indies, and southern Texas. The dis-

¹ *Arquivo da Sociedade d. Medicina e Chirurgia d. São Paulo*, 1910, 1, p. 2.

tribution of the disease in Panama is being investigated, for, as there is a disease among horses in the interior known as anthrax, there can be no doubt but that equine piroplasmosis is endemic in this region among native animals.

The probable transmitting agent.—*Dermacentor nitens*, the tropical horse tick of America, was described by Neumann in part from specimens taken from horses in San Domingo and Jamaica. In 1901, he listed it from Guatemala, Venezuela, and Porto Rico. It has been taken at Brownsville, Harlingen, and Kerrville,¹ Texas. Newstead² stated that *D. nitens* in Jamaica, 1908-9, was distributed fairly widely over the island, tho not an abundant species anywhere. He stated that it was rare apparently in the United States, but common on horses at San Domingo. Newstead found it almost exclusively confined to horses and mules, chiefly the former, occurring in little colonies inside the ear, altho found in other natural cavities of these animals. This is our experience in Panama, with the modification that mules, from the nature of their work on roads not frequented by native horses, are rarely infested, while saddle horses when used on the trails become infested.

Banks,³ in an interesting and prophetic note on *D. nitens*, states that he has taken this tick at Fort Bowie, Arizona, and that specimens from Haiti were in the Museum of Comparative Zoölogy. In noting morphological features he calls attention to the fact that this tick is strongly separated from all other species of the genus, the stigmal plate and impressed lines behind, with the male, being peculiar and noticeable. The very short palpi, shorter than the hypostome, he states, constitutes a peculiar character again seen in the cattle tick and, as in this species, *may indicate some habit connected with the dissemination of disease*.

Hooker,⁴ states that *D. nitens* is found in Texas from Brownsville to Corpus Christi and passes both molts upon the host, and records⁵ *D. nitens* from Texas, Arizona (?), Guatemala, Costa Rica, Cuba, Jamaica, San Domingo, Porto Rico, and Trinidad.

In Panama, *D. nitens* taken from a native horse at Chorrera

¹ Hunter & Hooker, *Bull.* 72, Bur. Entom., U.S. Dept. Agric., 1907.

² *Ann. Trop. Med. and Parasit.*, 1909, 3, p. 421.

⁴ *Jour. Econ. Entom.*, 1908, 1, p. 47.

³ *Bull.* 15, Bur. Entom., U.S. Dept. Agric., 1908.

⁵ *Ibid.*, 1909, 2, p. 404.

April 9 began ovipositing April 17 and continued each day until April 27, excepting April 23 and 24. Eggs were placed in a sunlit place and began to hatch May 10. All eggs were hatched and larvae began to swarm May 14. They could not under any circumstances be made to attach themselves to, and remain on, guinea-pigs.

Prophylaxis treatment.—The prevention of the occurrence of the disease in the United States requires that prohibition be placed on animals from infected zones entering regions where *D. nitens* is propagated.

Attempts to destroy ticks in a country like Panama are doomed to failure, for, while it might be possible to rid for a time certain pastures of ticks, the trails are simply alive with them.

For a remedy, Nuttall and Hadwen have recommended trypanblau, and according to Yakimoff, the disease has been treated successfully in Russia by Bielitzer with this drug.

PLATE 3.



FORMS OF *Piroplasma caballi* SEEN IN HORSE NO. 35.
Nos. 1-9, inclusive, illustrate phases in the process of subdivision.

A STUDY OF THE VIRUS OF RABIES, FREED FROM THE CELLS OF THE HOST AND FROM CONTAMINATING ORGANISMS.*

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I. INTRODUCTION.

Experimental work on rabies has largely been carried on with the virus as it occurs in the brain and spinal cord, where it is closely

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† This paper was presented before the Congress of Hygiene, Washington, D.C., September, 1912.

associated with the elements of the nerve tissue. Comparatively little has been done with the virus as it occurs in the salivary glands, where it is presumably in a more or less free state.

By the application of two new methods¹ we have been able to obtain a virus of rabies from the submaxillary glands in a comparatively pure state, freed from cells of the glands and unmixed with other organisms. Our work with this virus has been carried on with two objects in view: (1) the determination, if possible, of its nature, and (2) a comparison of this gland virus with the brain and cord virus.

If this comparison brought out marked physical differences, it seemed to us that this would suggest that the organism undergoes a cycle and exists in a different condition in the submaxillary gland from that in which it is found in the nervous system.

Of course, we have also used our pure virus for microscopical and cultural studies, but altho considerable work has been done in this direction, the results as yet have been inconclusive, and we are still continuing the experiments.

II. EXPERIMENTAL WORK.

METHODS OF OBTAINING THE VIRUS FROM THE SUBMAXILLARY GLANDS.

GLYCERIN EXTRACT VIRUS.

The submaxillary glands from dogs, dead of street rabies, were used in both methods. Several years ago, one of us² found that the virus of rabies could be extracted with glycerin from the submaxillary glands of rabid dogs.

The glands were removed from the dog's head under aseptic precautions. After removal of the fibrous sheath, they were immersed in sterile neutral glycerin and allowed to stand in the icebox for six to twelve days. This glycerin extract retained its virulence when kept in the icebox for a very long time, in one experiment 191 days. No further work was done on the extract due to the limitations of experimenting with a glycerin virus.

In our present experiments, we found that altho virus may be extracted from the intact glands, more was obtained when the glands were cut in eight to ten pieces, and this was always done in the following work. The clear supernatant glycerin slightly colored by hemoglobin was pipetted off. This was usually found to be uncontaminated, and microscopical examination showed no formed cellular elements.

By our present method, dialysis through collodion sacs, we are able to get rid of

¹ Poor and Steinhardt, *Jour. Infect. Dis.*, 1913, 12, p. 202.

² Poor, *Proc. New York Path. Soc.*, 1906, 6, p. 85.

glycerin and have our virus uncontaminated in any fluid we select. All the operations are carried on under aseptic conditions. The sac is sterilized, the glycerin extract contains no contaminating organisms, and as the collodion membrane does not permit the usual bacteria to pass through, the fluid in which the sac is placed for dialysis need not be sterile.

The dialysis of the glycerin takes place very rapidly. In running physiological salt solution, Ringer's solution, or distilled water, one and one-half hours are usually sufficient for the removal of the glycerin from four or five cubic centimeters of the virus. After dialysis, the virus is diluted, the dilution varying with the amount of water the glycerin has previously extracted from the tissue.

If a concentration is desired, the collodion sac may be used as a filter, as according to our experiments this virus does not pass through it, and, therefore, as much of the fluid as is desired may be removed. It is possible that a certain amount of the virus adheres to the side of the sac, so the concentration cannot be computed exactly. As shown by one of us,¹ proteins and probably salts in solution gradually pass through the sac after absorption reaches a certain degree, but the protein and salt contents of the fluid within the sac will be somewhat concentrated as the water passes through most rapidly. This fact must be borne in mind in interpreting some of the results of concentration.

The technic used for making collodion sacs was that described by Novy² and modified by Gorsline.³ The sacs were made over a tube, which had a small opening in the bottom. This tube was turned several times in dilute collodion, and this collodion, when partially dried, was stripped off by forcing water through the tube. A glass tube was then inserted into the neck of the sac, and the junction strengthened by a ligature or coating of collodion.

The sacs were filled with distilled water, the tubes plugged with cotton, autoclaved at 105° C. for 15 minutes, allowed to cool, then emptied, immersed in sterile water and subjected to pressure (about three inches of mercury). If there was no evidence of leakage the sacs were used. The sacs were without flaws or air bubbles. For dialysis they were thick; for filtration thin. The virus obtained in this manner will be referred to as the *glycerin virus*.

THE ASPIRATION VIRUS.

The glands after being cut in eight to ten pieces were placed in distilled water and subjected to a vacuum of 29 inches of mercury for an hour. The pieces of glands were then pressed in an ordinary meat press and the expressed fluid centrifuged. After centrifugation the supernatant fluid was pipetted off and added to the water in which the glands were aspirated. The aspiration fluid alone was found to contain the virus, but more was present on the addition of the pressed-out juice. By this method a suspension of the virus was obtained in distilled water which was stronger than the glycerin virus, but which was not sterile and which contained fragments of gland cells, blood cells, etc. Subsequent experiments showed that a Berkefeld filter held back bacteria and formed tissue elements, but the virus passed through. Thus by filtration we were able to get a sterile virus. This virus will be referred to as the *aspiration virus*.

¹ Steinhardt, *Jour. Infect. Dis.*, 1910, 7, p. 675.

² *Laboratory Work in Bacteriology*, Ann Arbor, 1899, p. 499.

³ *Contrib. Med. Res.*, Ann Arbor, Mich., 1903, p. 390.

TABLE 1.
VIRULENCE TESTS OF ASPIRATION GLAND VIRUS (STREET RABIES).

Number of Test	Guinea-Pigs Inoculated	Number Infected	Individual Incubation	Average Incubation
1.....	3	3	Days 8 9 8	Days 8 1-3
2.....	2	2	8 10	9
3.....	2	2	9 9	9
4 (Two sets of glands aspirated together) ..	3	3	9 8 8	8 1-3
5 (Four sets of glands aspirated together) ..	2	2	8 8	8
6 (Two sets of glands aspirated together) ..	2	2	10 10	10
7 (Three sets of glands aspirated together)	3	3	16 19 25	20

VIRULENCE TESTS OF THE GLYCERIN EXTRACT VIRUS.

Glycerin extracts of the submaxillary glands from 45 rabid dogs (street virus) were tested for virulence. The brains of most of these dogs showed Negri bodies. As Table 2 shows, 33 of the extracts were virulent or 73.33 per cent; the inoculated guinea-pigs developed rabies with incubation periods ranging from 8 to 82 days. Of the non-virulent extracts the longest period in which the animals were kept was 69 days. This is unfortunate as we now realize that had we kept the animals longer, more cases of rabies might have developed.

In a few cases where the smears of the fresh brain showed no positive Negri bodies, the gland extract, either glycerin or aspiration virus, proved virulent with a short incubation period, shorter than the brain inoculation. Therefore, occasionally this virus may be used for diagnostic methods, especially in those cases in which the brain is not fresh, and with the aspiration virus of the glands the Berkefeld filtrate can be used.

The glycerin extract was diluted with twice the amount of salt solution and about one-third of a cubic centimeter was inoculated subdurally into each guinea-pig. Altho the guinea-pigs showed clinical rabies, the brain smears were always examined for Negri

TABLE 2.

VIRULENCE TESTS OF GLYCERIN EXTRACTS OF SUBMAXILLARY GLANDS OF RABID DOGS (STREET VIRUS).
VIRULENT EXTRACTS.

Number of Test	Number of Days in Glycerin	Number of Guinea-Pigs Inoculated	Number Infected	Individual Incubation Period (Onset of Symptoms)	Average Incubation Period
1.....	14	3	3	Days 8 13 14	Days 11 2-3
2.....	16	2	2	11 11	11
3.....	10	3	2 (1 died 24 hrs. after inoculation; not rabies)	10 11	10 1-2
4.....	4	3	3	14 10 10	11 1-3
5.....	10	3	3	16 18 18	17 1-3
6.....	4	3	3	10 10 10	10
7.....	10	3	3	10 12 14	12
8.....	13	3	2 (1 died in 4 days; not rabies)	13 13	13
9.....	6	3	2 (1 died in 7 days; not rabies)	55 65	60
10.....	6	3	2 (1 dead in 6 days; not rabies)	13 13	13
11.....	8	3	3	7 16 17	13 1-3
12.....	8	3	3	9 11 11	10 1-3
13.....	5	3	3	13 13 22	16
14.....	6	3	2 (1 discharged)	15 21	18
15.....	6	3	3	21 21 21	21
16.....	7	3	1 (1 dead in 1 day; 1 dead in 6 days; not rabies)	82	82
17.....	7	4	1 (2 dead in 4 days; not rabies 1 discharged after 82 days)	34	34
18.....	7	3	2 (1 dead in 4 days; not rabies)	12 20	16
19.....	7	3	1 (2 dead in 4 days; not rabies)	12	12

TABLE 2—Continued.

Number of Test	Number of Days in Glycerin	Number of Guinea-Pigs Inoculated	Number Infected	Individual Incubation Period (Onset of Symptoms)	Average Incubation Period
20.....	7	3	3	18 18 19	18 1-3
21.....	7	3	2 (1 discharged)	13 13	13
22.....	7	3	1 (1 dead in 4 days; 1 dead in 5 days; not rabies)	15	15
23.....	13	2	1 (1 dead in 5 days; not rabies)	14	14
24.....	6	3	1 (1 dead in 2 days; 1 dead in 4 days; not rabies)	16	16
25.....	8	3	2 (1 dead in 24 days; not rabies)	10 12	16
26.....	6	2	1 (1 discharged in 180 days)	21	21
27.....	7	3	2 (1 discharged)	10 10	10
28.....	7	3	1 (1 dead in 25 days; not rabies. 1 discharged)	11	11
29.....	8	3	2 (1 discharged)	17 18	17 1-2
30.....	12	3	1 (2 discharged after 138 days)	14	14
31.....	14	3	2 (1 discharged)	9 10	9 1-2
32.....	12	3	1 (1 missing after 4 days. 1 discharged 36 days)	18	18
33.....	14	3	1 (2 missing after 6 days)	18	18

bodies and if not found reinoculations were made into fresh guinea-pigs. The possibility of a contaminating infection giving paralytic symptoms closely resembling rabies was brought to our notice in some of our animals and a report of that work will be published later.

That a single glycerin extraction may not remove all the virus from the glands was shown by the following experiment. In the virulent extracts, the glycerin extract of Virus 15 was withdrawn from the glands after six days and fresh glycerin added. This second glycerin extract was removed after eight days. The first extract gave rabies to guinea-pigs (3 in each set) with an average incubation period of 21 days, while the second gave rabies with an

NON-VIRULENT EXTRACTS.

Number of Test	Number of Days in Glycerin	Number of Guinea-Pigs Inoculated	Number Infected	Date Discharged
1	50	2	None	Days
2	23	2	None	37
			(1 dead in 18 days; bodies found; inoculation negative)	No Negri Reinoculation negative
3	14	3	None	60
4	4	3	"	53
5	14	3	"	47
6	7	3	"	47
7	13	3	"	47
8	14	2	"	69
9	8	3	"	2 discharged in 85 days
10	6	3	None	2 discharged in 85 days
			(1 died in 64 days; not rabies)	
11	10	3	None	2 discharged in 138 days
			(1 dead in 26 days; not rabies)	
12	14	3	None	172

average of twenty-four and two-thirds days. This showed but a slight dilution of the virus.

VIRULENCE TESTS OF SUBMAXILLARY GLANDS WHOSE GLYCERIN EXTRACTS ARE NEGATIVE.

That the action of glycerin on the glands is mainly that of sucking out the virus from the tubules is seen from the following experiment. The glands from two rabid dogs had given a non-virulent glycerin extract. They were removed from the glycerin and ground up in a mortar with salt solution, a difficult operation owing to the tough and slippery condition of the glands. These emulsions, extracted in the icebox overnight, proved to be virulent, giving rabies with an average incubation period of 29 days in one case, and of 12 days in the other. In another set of glands in which the glycerin extract had an incubation period of 56 days, the ground-up glands gave rabies in 10 days.

On the other hand, in some glands much virus was present in the ducts, as we have shown by a second extraction of virus with fresh glycerin giving rabies with a short incubation, confirming the fact of the variability of the virulence of the saliva of rabid animals.

VIRULENCE TESTS OF GLYCERIN EXTRACTS OF OTHER ORGANS.

Rabid brains.—Glycerin extracts were made of 11 fixed virus brains (rabbit) of which only four were virulent, and the glycerin

extracts of four street virus brains (dog) were all non-virulent (see Table 3), showing in all only four virulent extracts from 15 brains, that is 26.66 per cent virulence compared with 73.33 per cent of the glycerin gland extract.

When one considers that the brain, at least for fixed virus, is the culture medium for the rabies organism, which is present in much greater numbers in this situation than in the glands, one

TABLE 3.
VIRULENCE TESTS OF GLYCERIN EXTRACTS, RABID BRAINS OF RABBIT FIXED VIRUS AND DOG STREET VIRUS.

Number of Test	Material Extracted	Number of Days in Glycerin	Filtered	Guinea-Pigs Inoculated	Number Infected	Average Incubation Period	Discharged
1.....	Fixed virus rabbit brain	9	Not filtered	2	None	None	Days 36
2.....	" " " "	12	Not filtered	3	"	"	" 53
3.....	" " " "	12	Through cotton	3	"	"	" 53
4.....	" " " "	9	a Not filtered	3	2	11½ days	1 in 46
			b Through 1 layer of filter paper	3	1	13 "	2 in 41
5.....	" " " "	9	a Not filtered	3	2	9 "	1 in 46
			b Through 1 layer of filter paper	3	2	10½ "	1 in 41
6.....	" " " "	11	Through 1 layer of hardened filter paper	3	2	8 "	1 in 46
7.....	" " " "	11	Through 1 layer of hardened filter paper	3	None	46
8.....	" " " "	10	Through 1 layer of hardened filter paper	3	1	8 "	"
9.....	" " " "	10	Through 1 layer of filter paper	3	None	"
10.....	" " " "	10	Through 1 layer of filter paper	3	"	"
11.....	" " " "	10	Through 1 layer of filter paper	3	"	"
12.....	Street virus (dog) brain	16	Through cotton	3	"	33
13.....	" " " "	16	Through cotton	3	"	"
14.....	" " " "	16	Through 1 layer of filter paper	3	"	"
15.....	" " " "	12	Through 1 layer of filter paper	3	"	"

would expect a greater number of virulent brain extracts, unless, as is undoubtedly the case, the virus is here intimately associated with the nerve cells. Indeed the virulent extracts from the brain virus might be accounted for by minute particles of the brain substance being torn off by the action of the glycerin (the brain being a more delicate tissue than the gland) or by the extraction of the virus from the blood and lymphatic vessels and spaces, and as we have shown, the virus, even in the submaxillary glands, is not always in an extractable state.

In one case where the brain of a rabid dog (street virus) showed Negri bodies, part of the Ammon's horn, suprarenal capsule, and sciatic nerve were immersed in glycerin; the extracts were tested for rabies and all were non-virulent. In three cases of street rabies the glycerin extract of the pancreas was non-virulent.

These experiments are too few from which to draw conclusions; they were carried out to try the glycerin extraction on other organs which have been found virulent in rabies. Whether the aspiration method would have shown the virus to be in an extractable condition in these organs is to be decided by further experiments.

COMPARISON OF THE EFFECT OF GLYCERIN ON DIPHTHERIA AND TUBERCLE BACILLI.

Emphasis is usually laid on the resistance of the parasite of rabies to the action of glycerin, and this resistance is frequently considered to be opposed to the virus being of a bacterial nature.

We have made comparative tests with cultures of tubercle and diphtheria bacilli. Ten cubic centimeters of pure sterile glycerin were put on the cultures (agar and egg media) so that the growth was completely immersed. These were placed in the icebox for two weeks in the dark.

The supernatant glycerin from the diphtheria culture was dialyzed and planted in broth; incubation at 37.5° C. gave a vigorous growth of diphtheria organisms. The examination of the stained slides showed the bacilli in glycerin to be perfectly stained altho somewhat small. After dialysis, the stained slides from the non-incubated fluid showed bacilli indistinguishable from those from a fresh culture.

On cultures of bovine tubercle bacilli on egg media about 10 c.c. of glycerin was added so that the growth was more than covered and placed in the icebox in the dark. Three weeks later the supernatant glycerin was clear; there were no visible particles in it. Three cubic centimeters of this glycerin were dialyzed in running 0.8 per cent salt solution in a collodion sac for two hours. This dialyzed fluid was inoculated into guinea-pigs and produced general tuberculosis in two to three weeks.

After an interval of 13 months in the icebox, a portion of the growth of the same cultures, still covered by glycerin, was removed

emulsified and injected into guinea-pigs. These died of general tuberculosis, showing that the tubercle bacilli had not lost their virulence even after being acted upon by the glycerin for 13 months. The staining showed no diminution in the acidfastness of the organisms, and transfers gave growth on egg medium.

These two cultures, diphtheria and tubercle bacilli, are organisms which are not killed by drying, and as the action of glycerin is largely the withdrawal of water from that with which it is in contact, our results are not surprising. We find that these bacteria are not destroyed by glycerin, the tubercle bacillus having a resistance as great as the virus of rabies. This resistance, therefore, cannot be considered as necessarily indicating a protozoal virus.

We are now starting experiments on the action of glycerin in the cold and dark on other organisms, that may possibly have some bearing on the virus of rabies.

COMPARISON OF BRAIN AND GLAND VIRUS.

There are various hypotheses as to the nature of the virus of rabies. Some investigators consider it of bacterial origin with possible spore formation, while others favor a protozoal theory with a definite cycle. There are no Negri bodies in the gland virus though they exist in the brain. To test the question of a possible difference in the physical state of the virus in the two situations, brain and gland, differences which might throw light on the supposed cycle or spore formation, comparative experiments were made on viability, virulence, action of glycerin, the effect of rapid drying, of heat, of combined drying and heat, of dialysis, and of some chemical agents. In no instance was there any definite difference in the reaction of the two viruses to any of the tests, showing that as far as our work goes at present, it all indicates a similar physical condition of the virus of rabies in the gland and in the brain.

Viability.—Gland virus, aspirated in distilled water, was kept in an icebox at 7–8° C. for 15 days, at the end of which time it had become offensive through bacterial contamination. It was then filtered through a Berkefeld V candle, a filtrate being obtained which produced rabies in guinea-pigs with an incubation of 14 days.

This filtrate kept at room temperature and exposed to light for 45 hours had lost its virulence completely.

An emulsion of fixed virus in distilled water exposed to the same conditions for the same length of time was markedly weakened, the incubation of the injected animals being increased to 10 days, five days longer than the controls. Continuing the exposure to light and room temperature for 96 hours destroyed the virulence of the brain emulsion.

This experiment gives but a rough comparison as there were undoubtedly many more organisms present in the fixed virus emulsion than in the gland virus filtrate, and, as is known, the number of organisms present, and the amount of protein and salts in the emulsion greatly modify the results.

In another experiment the Berkefeld filtrate from an aspiration gland virus kept in the icebox for nine days had lost its virulence while the unfiltered virus kept in the same manner for the same length of time and then filtered proved virulent. This is in accord with the experiments of Di Vestea¹ on brain virus. He found that the filtrate of fixed virus died out in the icebox with'in a week, while the original unfiltered virus retained its strength for a long time.

Di Vestea also found the filtrate to be more easily destroyed by heat and glycerin, and from these results he concludes that the filtrate does not contain the entire rabic virus, that the virus is of a protozoal nature, of which only the young forms are filterable, and that these young forms are more easily destroyed by heat and glycerin and conservation. Di Vestea's facts are undoubtedly correct but there is another and simpler interpretation for them. As we have said before, in all comparative tests, variations in the number of organisms present and the concentration of proteins and salts of the material tested vary the results greatly. There are undoubtedly fewer organisms in a filtrate than in the original unfiltered virus and as shown by one² of us unless one filters a sufficient amount to saturate or coat the filter, the filtrate will contain considerably less proteins than the original solution. The fact that Di Vestea found his filtrate less resistant than his original

¹ *Ann d'ig. sper.*, 1905, 15, p. 147; *La Med. Ital.*, 1904, 2, p. 241.

² Steinhardt, *Jour. Med. Research*, 1904, 13, p. 409.

emulsion and the confirmation of that fact in this work with gland virus is more probably explained by the fewer number of organisms and the lesser amount of proteins in the filtrate than by a protozoal cycle.

Comparative virulence.—The incubation period of the gland virus is usually much shorter than that of the brain in street rabies. Subdural inoculations in guinea-pigs with emulsion of dog's brain, street virus, generally produce rabies with an incubation period of 14 days, as compared with the usual eight to nine days with the gland virus. This difference in incubation period could be explained in several ways: (1) the lesser virulence of organisms in the brain virus (2) the lesser number of organisms in the brain virus; (3) the presence of brain substances in the inoculations with this virus: this brain substance might act either directly or by causing a slowness of absorption.

One experiment with the glycerin extract of a dog's brain, street virus, showed an incubation period of only nine days. As in our other glycerin extract tests a piece of brain of a rabid dog, street virus, was put in glycerin for six days and then some of the supernatant glycerin was removed. This glycerin was not filtered and contained any small particles of brain matter, so we could not consider it as an extract and omitted it from Table 3. Three guinea-pigs were inoculated with it using the same methods and dilutions as for the other extracts. Of these, one contracted rabies with an incubation of nine days, the other two were negative and discharged 108 days later. Thus at least for this one case the street brain was as virulent as the gland extract. As yet we have not made a further study of this question and until more work has been done, no statement can be made as to the comparative strength of the street virus in the two situations.

The fixed virus is undoubtedly the stronger, judging from the shorter incubation period, even at great dilutions.

Action of glycerin.—The action of glycerin, according to Marie,¹ in destroying the virus of the rabid brain is a slow one if the mixture is kept at icebox temperature and is dependent on the amount of brain tissue, and it is difficult to fix the limits of this action.

¹ *L'étude expérimentale de la rage* (Dorin et Fils, editors), 1909, p. 124.

Poo¹ found, as quoted before, that under similar conditions the gland extract retained its virulence a long time. In one case at the end of 191 days, guinea-pigs inoculated with the glycerin extract showed rabies after 12 days' incubation.

In the present experiments, we have found a gradual weakening of the glycerin gland virus, kept in the icebox. This weakness was shown by a prolonged incubation period or complete avirulence.

In one instance, the glycerin that had been on the glands for 10 days was inoculated into three guinea-pigs. It infected all with an average incubation period of 12 days. The same extract kept on the gland in the icebox, 37 days later infected two out of three guinea-pigs with an average incubation of 19 days.

In one case we tested a virulent glycerin extract virus after six months' preservation in the icebox and found it had lost its virulence.

When kept in the icebox, the action of glycerin on the virus of rabies in the brain or in the gland, altho a variable one, brought out no difference between the virus from these two sources.

Rapid drying.—It is an established fact that brain virus if dried rapidly retains its virulence. That gland virus under similar conditions is equally as resistant is shown by the following comparative experiment.

As Harris and Schackell¹ state that it is the concentration of salts in the drying which causes the destruction of the rabies virus, to partially eliminate this factor, a preliminary dialysis in distilled water was done with the brain virus. An emulsion of fixed brain virus was made with distilled water put in a collodion sac and dialyzed in running distilled water for two hours. Part of this dialyzed fluid was kept in the icebox as control. The remainder was spread in a thin layer and dried in a vacuum of 29 inches of mercury over sulfuric acid. After 16 hours the drying was complete and the material was rubbed up in salt solution to its original volume. Subdural inoculations in guinea-pigs with the same doses of this and the control emulsion produced rabies in five to six days in both cases.

The glands from two rabid dogs (street virus) were aspirated

¹ *Jour. Infect. Dis.*, 1911, 8, p. 47.

separately. These two aspiration gland viruses were put under the same conditions as the brain virus.

The results here also showed no destruction of the virus, as inoculations with both the dried and the control gland virus gave rabies to guinea-pigs in nine and one-half days.

TABLE 4.
EFFECT OF RAPID DRYING ON EMULSIONS OF FIXED BRAIN VIRUS AND ASPIRATION GLAND VIRUS (STREET).

Number of Test	Material Inoculated	Guinea-Pigs Inoculated	Individual Incubation	Average Incubation
			Days	Days
1 (Control fixed virus) ..	Fixed virus brain emulsified in distilled water and dialyzed	3	5 5 7	5 2-3
2 (No. 1 dried)	5 c.c. of No. 1 dried in vacuum, then emulsified to original volume with salt solution	3	5 5 7	5 2-3
3 (Control fixed virus) ..	Fixed virus brain emulsified in salt solution	2	5 5	5
4 (No. 3 dried)	5 c.c. of No. 3 dried in vacuum, then emulsified to original volume with distilled water	2	1 dead in 7	7
5 (Control gland virus A)	Aspiration gland virus (street) A	2	8 10	9
6 (No. 5 dried)	Same as gland virus A. Dried in vacuum for 10-15 hours, then emulsified with salt solution	3	9 13 10	10 2-3
7 (Control gland virus B)	Aspiration gland virus (street) B	3	9 9 10	9 1-3
8 (No. 7 dried)	Same as gland virus B. Dried in vacuum for 10-13 hours, then emulsified with salt solution	3	10 8 9	9

Effect of combined rapid drying and heat.—An emulsion of fixed virus and a glycerin gland virus was dialyzed in distilled water for an hour. Very thin layers of each were evaporated to dryness while exposed to 37° C. for six hours. In each case the virus was killed while the controls were fully virulent. No difference in the nature of the two could be detected by this means.

Effect of heat.—In regard to the thermal death point of the virus in rabid brain emulsions, Marie¹ states that the length of time required to weaken and finally destroy the virus by heat varies according to the thickness of the emulsion, or to express it otherwise, according to the number of specific germs present.

¹ *Loc. cit.*

It was not possible to standardize the emulsions for comparison, but our experiments showed no marked difference between the fixed brain virus and gland street virus, altho the original gland virus was the weaker as shown by the longer incubation when inoculated into guinea-pigs.

Exposure for 10-15 minutes to 48-50° C. weakened both viruses, while the same temperature for 30 minutes killed the gland virus, and greatly weakened the fixed brain virus, only one guinea-pig out of three inoculated developing rabies and that with a prolonged incubation.

Both viruses were destroyed when kept at 60° C. for 30 minutes as shown by Table 5.

TABLE 5.
EFFECT OF HEAT.

Number of Test	Material Inoculated	Heated	Guinea-Pigs Inoculated	Individual Incubation	Average Incubation
1.	Glycerin extract gland virus dialyzed in distilled water for one hour (Control)		3	12 days 11 " 11 "	Days 11 1-3
2.	Fixed virus brain emulsion dialyzed same as above		3	5 " " " 1 dead in 1 day; not rabies	4
3. . . .	Fixed virus brain emulsion	48-50° C. for 15 min.	3	5 days 6 " 6 "	5 2-3
4. . . .	Glycerin extract gland virus dialyzed for 1½ hours in salt solution	48-50° C. for 10 min.	2	1 dead in 60 days 1 dead in 10 days; not rabies	60
5.	Fixed virus brain emulsion	48-50° C. for 30 min.	3	9 days; 2 discharged in 62 days	9
6.	Glycerin extract gland virus dialyzed for 1½ hours in salt solution	48-50° C. for 30 min.	3	Discharged in 62 days
7.	Glycerin extract gland virus dialyzed for 1½ hours in salt solution	60° C. for 10 min.	3	Discharged in 48 days
8. . . .	Fixed virus brain emulsion dialyzed same as above	60° C. for 30 min.	3	Discharged in 48 days
9.	Glycerin extract gland virus dialyzed for 1½ hours in salt solution	60° C. for 30 min.	3	Discharged in 48 days

Effect of chemicals.—The chemicals tested on the brain, centesimal emulsion of fixed virus, and the aspiration gland virus were bichlorid of mercury, hydrochloric acid, and magnesium dioxid.

With none of these was there any marked difference in the resistance of the two viruses. A 1-1000 solution of bichlorid of mercury killed both viruses. A 1-1000 solution of hydrochloric acid killed the brain virus and markedly weakened the gland virus, only one guinea-pig out of three contracting rabies with a prolonged incubation. Magnesium dioxid was tried because of its oxidizing effect. In the strength of one-fourth of a gram to four cubic centimeters of the virus, it produced but little effect.

Effect of dialysis.—Novy and Knapp¹ have shown that dialysis kills fixed rabies virus apparently through the extraction of certain salts and that the time required depends on certain factors such as the thickness of the emulsion, rate of the flow of water, etc. Our results were similar to theirs, tho not identical, probably owing largely to the fact of our using much stronger emulsions. We found, e.g., that the dialysis of two cubic centimeters of either brain or gland virus in 200-300 c.c. of standing water had but little effect after 18 hours, but that dialysis of the gland virus in running water for 18 hours killed it. Brain virus on the other hand, while it was markedly weakened, was not completely killed. Dialysis for 43 hours killed the fixed virus emulsion used by us. There were many more tissue elements as well as organisms in the brain virus emulsion used than in the glycerin gland virus which undoubtedly accounts for the longer time required for killing the former virus.

Effect of concentration.—Some of the filtrate from a Berkefeld V candle was evaporated in a vacuum over H_2SO_4 to one-fifth its bulk—the operation requiring four hours. The control in a narrow tube sealed with paraffin was placed in the apparatus so that conditions of light and temperature would be the same. Three guinea-pigs were inoculated from the concentrated virus as well as from the control and also the concentrated virus diluted to its original volume. The result was that while the pigs inoculated with the control died with an average incubation of 20 days, those from the concentrated virus developed with an incubation of 32 days, while only one pig from the concentrated virus subsequently diluted, developed after 20 days.

This suggests a harmful concentration of salts or proteins, such

¹ *Jour. Infect. Dis.*, 1906, 3, p. 291.

as is believed by Harris¹ to cause the reduction of virulence in the Pasteur cords.

TABLE 6.
EFFECT OF DILUTION AND CONCENTRATION.

Number of Test	Material Inoculated	Number of Guinea-Pigs Inoculated	Number Infected	Individual Incubation	Average Incubation
1a.	Control virus aspiration gland No. 1	3	3	Days 9 9 8	Days 8 2-3
1b.	Above virus diluted 1 to 4	3	3	8 9 11	9 1-3
2a.	Aspiration gland virus No. 2	2	2	8 8	8
2b.	Above virus diluted 1 to 8	4	4	12 9 9 9	9 3-4
3a.	Aspiration gland virus No. 3	2	2	10 10	10
3b.	Aspiration gland virus No. 3 diluted 1 to 10	4	3	9 11 17	12 1-2
3c.	Aspiration gland virus No. 3 diluted 1 to 20	4	4	9 11 13 15	12
4a.	Aspiration gland virus No. 5	2	1	8 (1 dead in 2 days; not rabies)	8
4b.	Aspiration gland virus No. 5 diluted 1 to 30	3	3	10 9 12	10 1-3
5a.	Aspiration gland virus No. 6	3	3	16 19 25	20
5b.	Aspiration gland virus No. 6 concentrated over sulfuric acid in vacuum (5.5 c.c. to 1.2 c.c.)	3	3	19 33 45	32 1-3
5c.	Same virus as 5b. After concentration diluted to original volume with salt solution	3	1	20	20

Effect of filtration.—In 1903 Remlinger² first showed that fixed virus was filterable. He emulsified the entire brain of a fixed virus rabbit in 400 c.c. of water and filtered the emulsion through a Berkefeld V filter under pressure. Ten rabbits were inoculated with from one-half to one cubic centimeter each of the filtrate.

¹ *Jour. Infect. Dis.*, 1912, 10, p. 369.

² *Ann. de l'Inst. Pasteur*, 1903, 17, p. 834.

Three of these rabbits or 30 per cent developed rabies. The following year Marie,¹ using three different Berkefeld candles, was unable to pass the virus through in any instance. In the same year, 1904, however, Bertarelli and Volpino² successfully filtered fixed virus through a Berkefeld V filter five times out of seven trials. They failed to pass it through a Chamberland F. Celli and du Blasi³ had similar results. In 1904 also Di Vestea⁴ made extensive experiments on filtration. He used fixed virus and prepared the emulsions with great care, emulsifying the brains with the aid of quartz sand. He filtered with from two to three atmospheres pressure and inoculated his animals with large amounts of the filtrate, i.e., one-half to one cubic centimeter each. His results were as follows:

	Experiments	Virulent Filtrates	Per Cent
Berkefeld V.....	19	14	75
Berkefeld N.....	1	1
Chamberland F.....	8	2	25
Chamberland B (Filtrate from Chamberland F).....	1	0
Maasen Filter (Filtrate from Berkefeld).....	1	1

The literature contains very little regarding the filtration of gland virus. In one instance a virulent filtrate was obtained by Bertarelli and Volpino, who passed it through three layers of paper. It is also recorded that the virus in human saliva was passed in one instance through a Berkefeld candle.

Our experiments were made with Berkefeld V and N, Chamberland F and B, and with a collodion filter. The aspiration virus from the glands of from two to four dogs were used in each experiment. The results were as follows:

	Experiments	Virulent Filtrates	Per Cent
Berkefeld V.....	4	4	100
Berkefeld N.....	1	1
Chamberland F.....	4	3	75
Chamberland B.....	2	0
Collodion Sac.....	2	0

¹ *Loc. cit.*

² *Centralbl. f. Bakteriöl., I Orig.*, 1904, 37, p. 51.

³ *Ann. d'ig. sper.*, 1904, 3, p. 603.

⁴ *Riv. d'ig.*, 1905, 16, p. 158; *La Med. Ital.*, 1904, 2, p. 241.

In the collodion sac and the first Chamberland B experiment, the virus unfortunately proved to be quite weak. In the second Chamberland B experiment, however, a strong virus was obtained, but inoculations with the filtrate failed to show symptoms of rabies after a lapse of six weeks.

In order to get some idea of the amount of virus held up by the filter, a series of dilutions of the fluid to be filtered was made as follows: dilutions of 1 to 4, 1 to 8, 1 to 10, 1 to 20, and 1 to 30. It was found that the average incubation of the guinea-pigs inoculated was increased in a surprisingly regular manner, according to the degree of the dilution of the virus. For example, the dilution of 1 to 4 gave an incubation of nine and one-third days, as against eight and two-thirds days for the undiluted virus, an increase of two-thirds of a day only. A dilution of 1 to 30 gave an incubation of ten and one-third days as compared with eight days for the original virus, an increase of two and one-third days (see Table 6). The average duration of incubation, then, may be taken as a rough index of the dilution of virus. All of the guinea-pigs inoculated with the Berkefeld filtrates developed rabies. That more of the virus passes through after saturation of the filter is seen from the following experiment. A small Berkefeld V candle was used. The first five cubic centimeters which passed constituted the first filtrate, the next 12 c.c., the second filtrate. The four guinea-pigs inoculated with the first filtrate developed rabies with an average incubation period of 21 days. Those with the unfiltered material diluted eight times developed in nine and one-half days and the undiluted control in eight days. Comparing the incubation of the pigs injected with the second filtrate, twelve and one-half days, with that of the pigs injected with the control diluted 1 to 8, nine and one-half days, it is fair to assume that in this case the virus was diluted by filtration more than eight times. Again in the finer Berkefeld, Grade N, the average incubation of the pigs injected with the second filtrate was fifteen and one-half days, and that produced by the virus diluted 1 to 20 was only eleven and one-half days. In a further attempt to determine the dilution caused by filtration, a Berkefeld V candle was shaved to about one-half its thickness. In this case a weak virus diluted 1 to 20 gave an incubation of 17

TABLE 7.
 FILTRATION THROUGH BERKEFELD V FILTER.

Number of Test	Material Inoculated	Number of Guinea-Pigs	Number Infected	Individual Incubation	Average Incubation
				Days	Days
1.	Glycerin extract gland virus (Control) dialyzed	5	2	12 18	15
2.	First 5 c.c. of filtrate of Test 1	5	0	Discharged 3 months later, not rabid
3.	Last (30 to 35 c.c.) of the filtrate of Test 1	5	1	56	56
4a.	Aspiration gland virus (4 sets of glands) control diluted 1 to 8	4	4	12 9 9 8	9½
4b.	Virus of Test 4 undiluted	2	2	8 8	8
5.	Virus of Test 4 passed through filter (first 5 c.c. of filtrate)	4	4	18 26 14 26	21
6.	Last 12 c.c. of the filtrate of Test 4	5	4	12 14 13 11	12½
7.	Aspiration gland virus (3 sets of glands) passed through the filter	3	3	16 19 25	20
8.	Virus of Test 7 kept in icebox 6 days, showed evidence of bacterial contamination, passed through filter	3	2	1 dead in 3 days; not rabies 14 15	14½

Tests 1, 2, and 3.—The glycerin extract of two sets of rabid glands was dialyzed in one and one-half hours and then passed through a new Berkefeld V filter with the aid of a vacuum pump. The first five cubic centimeters, called the first filtrate, were inoculated in guinea-pigs; the next 25 c.c. were discarded (used to coat or saturate the filter) and the last five cubic centimeters were called the last filtrate and inoculated into guinea-pigs. The original virus was very weak, only two out of five guinea-pigs contracting rabies in Control Experiment 1. The last filtrate was virulent but not the first.

Tests 4a, 4b, 5, and 6.—Aspiration gland virus of four sets of rabid glands, amounting to 50 c.c. (much hemoglobin and very cloudy), was passed through a new Berkefeld V filter with the aid of a vacuum pump. The first five c.c., called the first filtrate, were inoculated into guinea-pigs; the next 12 c.c., called the last filtrate, were also inoculated. The filter was then blocked. The last filtrate proved more virulent than the first.

Tests 7 and 8.—Aspiration gland virus of three sets of rabid glands which were kept in the icebox four to six days was passed through a new Berkefeld V filter¹ by the aid of a vacuum pump. The first 15 c.c. were used to saturate the filter and the following seven cubic centimeters used for the experiment in Test 7. In Test 8 part of the original unfiltered virus used in Test 7 which had remained in the icebox for six days and showed evidence of bacterial contamination was passed through a new Berkefeld V filter with the aid of a vacuum pump. Eight cubic centimeters were used to saturate filter and the following six cubic centimeters, clear but of a reddish color and offensive odor, were injected into guinea-pigs, and proved virulent.

¹ New filters, generally the small size, were used in all filtrate experiments. The filter was washed in distilled water, then boiled in 0.4 per cent salt solution for 20 minutes. Before filtration was started the filter was partially drained by the aid of the vacuum. All filters were tested with a suspension of bacteria, usually pyocyaneus or diphtheria and proven perfect.

days while the filtrate from the shaved filter gave 16 days, practically the same, so that it may be assumed that the shaved filter diluted the virus about 20 times. The filtrates from the Chamberland filters gave correspondingly increased incubation. Thus in one test, two out of four pigs inoculated developed rabies with an average incubation of 33 days, as against eight and one-third days for the control. In a second test two out of five pigs developed with an average incubation of 47 days as against 11 days for the control. The filters were tested with a suspension of pyocyaneus bacilli or other bacteria which was mixed with the virus to be filtered. In no case did the bacilli pass through.

Comparing our results with the filtration of gland virus, with those obtained by others in the filtration of the fixed brain virus, one may infer that the greater regularity with which the gland virus passes (that is, 100 per cent in the Berkefeld as against 75 per cent, and 75 per cent in the Chamberland F as against 25 per cent obtained by Di Vestea) is due to the freer condition of the virus in the glands. That the organism is not necessarily smaller in this location is seen from the fact that the fixed virus did pass the Chamberland filter, and even in one instance, according to Di Vestea, the Maassen filter.

TABLE 8.
FILTRATION THROUGH BERKEFELD N FILTER.

Number of Test	Material Inoculated	Number of Guinea-Pigs	Number Infected	Individual Incubation	Average Incubation
1.	Aspiration gland virus (2 sets of glands); control diluted 1 to 20	4	4	Days 13 14 10 9	Days 11½
2.	Virus of Test 1 filtered through Berkefeld N	4	4	14 16 16 16	15½

Aspiration gland virus of two sets of rabid glands was passed through a new Berkefeld N filter with the aid of a vacuum pump. The first 15 c.c. were used to saturate filter. The next 10 c.c. were tested in Test 2 and proved virulent.

A culture of *B. alvei* was used for the test of the filter, and the filter proved bacteria proof.

TABLE 9.
 FILTRATION THROUGH PASTEUR CHAMBERLAND F.

Number of Test	Material Inoculated	Number of Guinea-Pigs	Number Infected	Individual Incubation	Average Incubation
1.....	Aspiration gland virus (3 sets glands). Control	5	4	Days 8 10 9 12 1 dead in 2 days, not rabies	Days 9 3-4
2.	Virus of Test 1 passed through filter	5	1	26	26 (4 pigs discharged 4 months later, not rabid)
3.	Aspiration gland virus (4 sets glands). Control	2	1	11 1 pig disappeared	11
4.....	Virus of Test 3, first 12 c.c. filtrate, passed through small filter	5	2	46 48	47
5.....	Virus of Test 3, first 20 c.c. of filtrate, passed through large filter	6	1 dead in 10 days, not rabies *1 dead in 17 days	Other pigs discharged 4 months later, not rabid
6.....	Aspiration gland virus (2 sets glands). Control	3	3	9 8 8	8 1-3
7.....	Virus of Test 6 passed through large filter and first filtrate used	4	2	27 39	33
8.....	Virus of Test 6 passed through large filter and last filtrate used	5	1 dead in 5 days 1 dead in 40 days No Negri bodies seen	Other pigs discharged 4 months later, not rabid

* Guinea-pigs (4) inoculated from this brain died in two days. The brain was then put into glycerin and three days later three more pigs were inoculated from this material. Two died on the next day and the other was discharged four months later, not rabid.

Tests 1 and 2.—Aspiration gland virus of three sets of rabid glands passed through a new Chamberland F filter. Fifty cubic centimeters of the extract were put through the filter of which 35 c.c. were recovered and used in Test 2. Pyocyanus broth culture used for efficiency of filter, filtrate sterile.

Tests 3, 4, and 5.—Aspiration gland virus of four sets of rabid glands was divided and passed through two new Chamberland F filters, one small size and one large. Filtration with the aid of a vacuum pump. In Test 4, 12 c.c. were passed through small filter of which four cubic centimeters were recovered. In Test 5, 20 c.c. were passed through large filter and seven cubic centimeters recovered. Pyocyanus broth culture used for efficiency of filter, filtrate sterile.

Tests 6, 7, and 8.—Aspiration gland virus of two sets of rabid glands was passed through a new Chamberland F filter, large size, with the aid of a vacuum pump. The filtration was slow. The first five cubic centimeters to pass through, called first filtrate, were used in Test 7 and proved virulent. The next 15 c.c. were used to saturate filter and discarded. The last five cubic centimeters, constituting the last filtrate, were tested for virulence in Test 8 and proved non-virulent. A broth culture of diphtheria was used for the efficiency of the filter. Filtrate sterile.

TABLE 10.
PASTEUR CHAMBERLAND B FILTRATION.

Number of Test	Material Inoculated	Number of Guinea-Pigs	Number Infected	Individual Incubation	Average Incubation
1.....	Aspiration gland virus (5 glands from 3 dogs). Control	2	1	1 dead, not rabies. 7 days	7 days
2.....	Virus of Test 1 filtered through large Pasteur B filter	5	1 dead, 3 days } Not 1 " 10.4 " } ra- 1 " 107 " } bies	Other pigs discharged 4 months later, not rabid
3.....	Aspiration gland virus (Control) 1-20 dil.	3	3	12 days 12 "a 17 "	13 2-3 days
4.....	Virus of Test 3 filtered through large Pasteur B filter	5	Discharged 4 months later, not rabid
5.....	Virus of Test 1a, Table 11, filtered through small Pasteur B filter	5	Discharged 4 months later, not rabid

Tests 1 and 2.—Aspiration gland virus of five rabid glands from three dogs was passed through a new Pasteur B filter with the aid of a vacuum pump. Before filtration, 25 c.c. of the virus was mixed with one cubic centimeter of a broth suspension of pyocyanus and proved filter bacteria proof. The filtrate proved non-virulent.

Tests 3 and 4.—Aspiration gland virus diluted one to twenty in salt solution to which two loopfuls of a moist agar culture of pyocyanus had been added was passed through a new large Pasteur B filter with the aid of a vacuum pump. Seventy-five cubic centimeters were filtered through in 15 minutes, then 25 c.c. of undiluted virus were further filtered through. As the filter had not been completely drained in the beginning 115-120 c.c. were recovered, and, inoculated in the guinea-pigs, proved non-virulent.

Test 5.—Aspiration gland virus was filtered through a shaven Berkefeld V filter (used in Test 1a, Table 11). This filtrate was shown to be virulent. It was refiltered through a new Pasteur B filter, small size; about 12 c.c. were passed through of which six cubic centimeters were recovered and proved non-virulent.

TABLE 11.
FILTRATION THROUGH COLLODION SAC.

Number of Test	Material Inoculated	Number of Guinea-Pigs	Number Infected	Individual Incubation	Average Incubation
1a.....	Aspiration gland virus No. 1a filtered through shaven Berkefeld V filter. Control	3	2	Days 17 17	17 days
1b.....	Aspiration gland virus No. 1a filtered through a collodion sac	5	0	Discharged 3 months later; not rabid
1c.....	Aspiration gland virus No. 1, unfiltered, diluted 1 to 20. Control	3	3	12 12 17	13 2-3 days
1d.....	Same virus as No. 1c filtered through a collodion sac	5	0	Discharged 3 months later; not rabid

Tests 1a and 1b.—Part of the aspiration gland virus that was filtered through a shaven Berkefeld V filter was used in Test 1a. Then 3.5 c.c. of it were filtered through a collodion sac with the aid of a vacuum of 2.5" to 3." The filtration required two and one-half hours. The filtrate tested in 1b proved non-virulent.

Test 1d.—Dilution of virus 1 to 20 used in Test 1c filtered through under same conditions as 1b. Filtration for 3.5 c.c. took five and one-half hours. Filtrate non-virulent. The sacs were tested by pressure before and after filtration and no leakage found.

ATTEMPTS AT CULTIVATION.

In the first method tried, the spinal cord of a fixed virus rabbit four days after subdural inoculation was used. At this time the virus has reached the cervical end but has not extended to the lumbar extremity. The cord was quickly removed from the canal by the Oshida method, and blocked into previously sterilized paraffin of 46° melting point, the whole block being then immediately solidified in ice water. In this way a partial anaerobic condition was obtained, a condition considered by Marie to be desirable. At the end of 10 days not only had the virus not extended to the lower end, but it had died out in the cervical end.

Attempts were made to grow the virus or at least to get an idea of its viability, by making use of the technic of Harrison's method of tissue growing *in vitro*. It was found that apparently the brain cells retained their vitality at incubator temperature for several days, in one instance as long as 21 days. This was inferred from the perfect staining of the Nissl bodies. However, in spite of the ganglion cells remaining alive, small portions of the fixed virus brains imbedded in plasma, agar, or in simple saline solution, lost their virulence after seven days at 37° C.

On the other hand, in the only instance in which street virus brain was tried, it was found that the virulence was retained after eight days at 37° C.

Portions of normal guinea-pig brain inoculated with weak emulsion of fixed virus showed no virulence after six days. Normal brain was also inoculated in a similar manner with gland virus, but here also there was no evidence of virus at the end of six days. Unfortunately in this experiment a glycerin extract virus was used which proved to be very weak. From our subsequent work, as well as from clinical observations, we would conclude that the most promising virus to use for this purpose would be the strong aspiration virus freed from bacteria by passage through a Berkefeld filter. This would avoid the objection to brain virus for the cultivation of the rabic organism made by Marie on the ground that at incubator temperature the brain tissue develops substances detrimental to the virus.

MICROSCOPICAL EXAMINATION.

The glycerin extract dialyzed in distilled water or in salt solution, even after concentration through a collodion sac or by evaporation in vacuum, gave a product which, while containing the organism, showed remarkably little in the way of formed elements of any kind. The aspiration virus when filtered through a Berkefeld V candle showed many small granules, of which some were evidently from the secreting cells of the gland, as they were seen also in extracts from the normal gland. Many examinations were made of both of these viruses with the dark field (1-12 and 1-16 lenses and 4 and 8 ocular), also unstained in light field and stained by the various intensive and special methods. In all cases small granules easily seen in the unstained preparations and taking most of the stains with comparative ease were found; but these were found in the extracts of normal glands too, altho it occasionally seemed to us that there were more granules in the rabid extracts. Sometimes a ring was seen with a central point and, rarely, slender, slightly curved structures, suggesting cilia, were found. We were unable, however, to identify any of our findings as the specific organism.

ATTEMPTS AT AGGLUTINATION.

Anti-rabic serum was produced by immunizing guinea-pigs and rabbits with fixed virus, according to Marie's method. This serum was tested for its agglutinative properties against the rabid gland extracts. Since rabid nerve tissue of the rabbit was used as the antigen, and the rabid gland extract of the dog for the agglutination test, it was hoped we might get a specific agglutination of the organism of rabies, with tissue reactions excluded, a result unobtainable by the use of rabid nerve tissue for both immunization and agglutination.

Preliminary experiments showed in the extracts of the glands of both normal and rabid dogs, an agglutination of granules, apparently greater in the rabid extracts than in the controls. The results were not sufficiently definite to warrant any conclusions. Further work is being carried on in this line and also with the opsonic technic and results will be given in a later paper.

III. SUMMARY AND CONCLUSIONS.

By our two new methods, glycerin extraction and aspiration, we have been able to obtain the virus of rabies from the submaxillary glands, free from the cells of the host and from contaminating organisms. We were thus able to study the virus in a fairly pure state and to compare it with the virus as it exists in the nervous tissue.

Forty-five glycerin extracts of the submaxillary glands of dogs (street rabies) were tested for virulence by subdural inoculations in guinea-pigs. Thirty-three or 73.33 per cent were virulent, the average incubation periods ranging from eight to 82 days. A single extraction of glycerin did not always extract all the virus, for in some cases tested, a second glycerin extract of the same glands also proved virulent. An emulsion from glands which had given a non-virulent extract was virulent, showing the action of the glycerin in extracting the virus to be largely mechanical, a sucking out of the virus from the tubules and ducts.

Glycerin extracts of 11 fixed virus brains (rabbit) were virulent in only four instances, while four glycerin extracts of street virus brain (dog) were all non-virulent, showing in all four virulent extracts from 15 rabid brains, that is, 26.66 per cent virulence as compared with 73.33 per cent of the glycerin gland extract. Considering the great virulence of fixed virus brains, one would expect a greater number of virulent glycerin brain extracts. The result, however, need not necessarily argue a difference of form of the virus in this situation, but rather indicates that the virus is here more intimately associated with the nerve cells, while in the glands it is probably largely a drawing out from the ducts. The few virulent brain extracts might have been due to very minute particles of the brain substance, the brain being more friable than the gland, or to the extraction of the virus from the blood and lymphatic vessels and spaces.

A few glycerin extracts made from the suprarenal capsule, sciatic nerve, and pancreas from the dog (street virus) were all non-virulent.

The aspiration virus of the rabid submaxillary glands was always virulent, usually with an incubation period of from eight to nine days.

The resistance of the virus of rabies to glycerin so frequently taken as suggesting a protozoal origin has been found by us to be paralleled by diphtheria and tubercle bacilli. Cultures of these bacteria, which were subjected to glycerin in the cold and dark, diphtheria bacilli for two weeks, tubercle for 13 months, on dialysis and transplantation, gave vigorous growths. Their staining properties were not impaired, and the tubercle bacilli were fully virulent; the diphtheria culture was not tested for virulence. As the action of glycerin is largely that of the extraction of water or drying and both the cultures withstand drying tho neither has a spore stage, the results are not surprising.

We find, therefore, that these bacteria are not destroyed by glycerin, the tubercle bacillus having a resistance as great as the virus of rabies, which resistance cannot therefore be considered as necessarily indicating a protozoal virus.

In comparing the virus as it exists in the brain and in the gland, this fact must be borne in mind: with viruses which cannot be standardized slight differences in reactions to either physical or chemical agents are not to be interpreted as indicating a difference in their physical or biological state, as variations in the number of organisms present in the concentration of proteins and salts vary the results with the reactions of the different emulsions.

Comparative experiments on the brain and gland virus for viability, for the effect of rapid drying, heat, combined drying and heat, filtration, dialysis, action of glycerin, of magnesium dioxid, of bichlorid of mercury and of hydrochloric acid, have shown no sufficient difference to indicate either a protozoan cycle or a spore bacterial form in the virus of rabies in these two situations.

In our filtration experiments with the gland virus we obtained 100 per cent virulent filtrates with the Berkefeld filters and 75 per cent with the Pasteur Chamberland F filter. Di Vestea, working with brain emulsion, secured only 75 per cent positive filtrates from the Berkefeld filter and 25 per cent from the Pasteur Chamberland F. This difference in results need not argue a smaller form of the organism in the gland. It may be due to the virus being less intimately associated with the cells of the host in this situation, as was previously suggested by the fact of glycerin extract of

rabid brains being virulent in only 26.66 per cent as compared with the 73.33 per cent of virulence of the glycerin gland extracts.

Whether this would indicate that the chromatin elements of the Negri body may be the true rabic parasite, the outer portion being a degeneration product of the nerve cell, we are at present unprepared to say. The following points are to be considered in this connection. First, the structure known as the complete Negri body could not pass through the filter; second, some of the minute, inner cell-structures might pass; third, the small central structures might easily exist free in the gland and escape identification. In this connection, it is interesting to note that working in conjunction with Dr. R. A. Lambert,¹ we have been able to produce inclusions in ganglion cells, closely resembling the outer portion of Negri bodies, by allowing normal nerve cells to slowly degenerate in plasma at 37° C. according to Harrison's technic for the growing of tissues *in vitro*.

Many factors also are to be considered in judging the results of filtration. The question of the coating or saturation of filter is sometimes of great importance, even for the passage of proteins in solution as shown by one of us in complement filtration, and as our present work has shown in the Berkefeld filtrate. Therefore, in any question of filtration the amount filtered, the first or last filtrate tested, the dilution of the organism and of the foreign protein and the amount of salts in the fluid to be filtered, the pressure used, the possible concentration of the filtrate by evaporation, centrifugation, precipitation, or possible agglutination, all are important factors toward the end results. So the comparison of filtration experiments unless done under identical conditions cannot be taken as absolute proof of difference in size.

The fact of a virus passing through even the Chamberland Pasteur F filter does not mean that it is invisible with our present powers of magnification. The organism of peripneumonia, the classic example of a filtrable virus, readily passes through this filter but is easily seen by the use of the dark field and oil immersion.

Our attempts at cultivating the virus of rabies even with the *in vitro* method have not as yet been successful.

From our microscopic examinations we conclude that the virus

¹ *Jour. Infect. Dis.*, 1912, 11, p. 459.

of rabies as it exists in the submaxillary gland, altho possibly visible, is probably of no distinctive form. By the use of various staining methods, by frequent examinations of many viruses with both light and dark fields, with high magnification, no distinctive formed elements were seen. In all cases small granules were readily found. Occasionally there were rings with a central point and cilia-like structures, but these were also found in the control extracts of normal submaxillary glands. Occasionally it seemed to us that there were more granules in the rabid than in the normal extracts, but no conclusions could be drawn. Experiments on the possibility of obtaining a specific agglutination of the virus of rabies as it occurs in the gland extract from an antirabic serum, obtained by the injection of rabid brain, were tried. By this method it was hoped to exclude tissue reactions. In some cases it seemed as if there were greater clumping of granules in the rabid extracts than in the control normal extracts. Further work is now being carried on along this line and with the opsonic technic with the possibility of a specific phagocytic action locating the virus in the leukocytes. Until these experiments are completed no definite conclusions can be drawn concerning the granules.

In closing, we wish to emphasize the following points:

1. The advantage of using the gland secretions in the study of rabies because they contain the organism freed from tissue cells. This is an advantage for the microscopical study of the virus, for the study of its physical properties, and for its use in growing experiments.
2. The two easy methods for obtaining bacterially sterile virus from the glands.
3. The resistance of bacteria to glycerin which is as great for the tubercle bacillus as for the virus of rabies.
4. The regularity with which the gland virus passes the Berkefeld filter and the large percentage of virulent filtrates with the Chamberland Pasteur F filter.
5. The similarity in the nature of the rabies virus in the two locations of the brain and the glands as shown by the comparative effect of physical and chemical agents.

In conclusion, we wish to express our thanks to Dr. C. Zell, Frederick Jelinek, and Miss L. Alper, who assisted largely in the technical part of the work.

THE ORGANISM OF RABIES AND EXPERIMENTS IN ITS ARTIFICIAL CULTIVATION.*

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The following experiments were carried on with a hope of securing some evidence which might aid in establishing the true nature of the Negri bodies. It has been frequently observed that, in the earlier stages of rabies, the Negri bodies either may not be demonstrable at all, or they may be few and minute, while in animals which have died of the disease the bodies are larger and more numerous. To trace this development through successive stages if possible was the purpose of the first series of experiments.

Dogs were inoculated with street rabies by the direct injection of emulsion of rabid brain into a nerve trunk. Various nerves were employed, but most satisfactory results were obtained by introducing a long needle into the orbital cavity below the eyeball and making the injection at the point where the optic nerve emerges through the optic foramen, the operation being made under ether anesthesia. The animal was then observed closely and at the first sign of unusual restlessness or excitability, usually after 12 to 18 days, it was killed and the brain taken out under the most careful aseptic precautions. Sections about 3 millimeters in thickness were taken through Ammon's horn and adjacent tissue, and incubated in sterile dog serum in tubes with a layer of olive oil above the serum. The olive oil served the double purpose of preventing evaporation and producing partial anaerobic conditions. Before placing the brain tissue in the tubes a small bit of gray matter was cut from each section and a smear made as a control for future comparison. At intervals of 24 hours tubes were opened and specimens made and compared with their respective controls.

The chief difficulty lay in determining the stage at which the dog should be killed and the incubation *in vitro* begun. When this stage was chosen opportunely either no Negri bodies would be seen

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in the freshly stained preparation, or the forms would be very minute, ranging from 1 to 3 microns in diameter. Marked increase in both number and size was evident in 24 hours in most cases and the development continued until the tissues became so degenerated from incubation that satisfactory staining was difficult. After 48 or 72 hours the larger bodies attained a diameter of from 6 to 9 microns while definite increase in numbers was evident, and the smaller forms were present in abundance. The bodies showed the characteristic dark granules and staining properties, but greater irregularity of form was evident than the Negri bodies show under ordinary conditions. Controls of normal brain tissue incubated under the same conditions showed no forms which would be mistaken for Negri bodies by an experienced observer. Equally satisfactory results were obtained when the sections were incubated in Ringer's solution instead of serum.

These results lend additional support to the view that the Negri bodies are forms in the life cycle of a protozoan organism as against the view that they are the results of the reaction of the living nerve cells to the disease. The increase in the size and the number of the bodies perhaps might result from a progressive chemical change in the brain substance, but the notion that the bodies are the results of the reaction of the living cell toward the unknown parasite or its toxin, is hardly consistent with the results of this experiment.

Cultivation of the supposed organism was attempted as follows: the medium used was fresh brain matter taken from a normal dog under the most careful aseptic precautions. The brain substance was proved sterile by incubation and subcultures. The following media were tried:

Brain material under sterile dog serum and oil;

" " " " cerebrospinal fluid and oil;

" " " " Ringer's solution and oil;

4 parts plain agar+1 part brain substance thoroughly mixed;

1 part brain+9 parts salt solution, emulsified by shaking with glass beads, and filtered through a coarse Berkefeld filter.

This filtrate under a layer of sterile oil was used as a culture medium.

Inoculations were made with a drop of emulsified rabid brain substance, and the medium incubated at 38° C. for from 8 to 15

days, both aerobically and under strict anaerobic conditions produced by a combination of vacuum, displacement by hydrogen, and absorption of oxygen by KOH+pyrogalllic acid. Smears were then made and stained with rosanilin violet+methylene blue as described by Williams and Lowden.¹

Guinea-pigs were inoculated with the medium after incubation as a test for virulence. As a control to determine how far the original infective material might be responsible for virulence of the subcultures, a series of tubes containing salt solution under oil were inoculated and subcultures made, incubated, and tested for virulence in the same manner as the culture tubes. The tubes of brain agar and brain emulsion filtrate gave no encouraging results either anaerobically or aerobically. The tubes of brain substance in dog serum, cerebrospinal fluid, and Ringer's solution, however, gave some encouragement, and as the results in these three suspension media were practically the same, Ringer's solution was adopted as a medium and the use of dog serum and cerebrospinal fluid was discontinued. Strict anaerobic conditions were also discontinued as unnecessary and only the partial exclusion of oxygen by olive oil or paraffin oil was practiced.

In microscopic examination of the cultures many bodies were found which resembled Negri bodies, but due to the degenerated condition of the brain substance following incubation it was impossible to say that these might not be degenerated cell nuclei or other artifacts. This being the case reliance was placed mainly on the results of animal inoculation. This was performed by introducing a drop of emulsified culture material subdurally through a small opening drilled through the skull of a guinea-pig. The inoculation was made under ether anesthesia and surgical asepsis.

Now follows a summary of the results of the series of animal inoculations.

TABLE 1.

Transfer or Subculture	Series	Guinea-Pig Died in	Results
1.....	4	45 Days	Negri bodies found
2.....	4	13 "	" " "
3.....	4	7 "	Meningitis
4.....	4	8 "	"
5.....	4	12 "	Negri bodies found
6.....	4	10 "	" " "

¹ *Jour. Infect. Dis.*, 1906, 3, p. 452.

In the sixth generation occurred a contamination of staphylococcus. An attempt was made to get rid of this by emulsifying the material and passing it through a Berkefeld filter, using the filtrate for further inoculations. This filtrate when incubated showed forms which very closely resembled Negri bodies. A delay in securing sterile media occurred at this time, after which subcultures made from the filtrate were not virulent for animals. Whether the delay or the filtration caused the loss of virulence is not evident.

TABLE 2.

Transfer or Subculture	Series	Guinea-Pig Died in	Results
1.....	20	10 Days	Negri bodies
2.....	20	7 "	Meningitis
3.....	20	12 "	Negri bodies
4.....	20	5 "	Meningitis
5.....	20	24 "	Negri bodies

In both these series the controls, which consisted of normal salt solution under oil, inoculated and incubated in the same manner as the cultures, were virulent for guinea-pigs in the second subculture. Animal inoculations with subsequent transfers of the controls gave negative results in every case. This would indicate that enough of the original infective material might be present in the second or third subculture to cause rabies in the animals inoculated. The fact that cultures of the virus in brain medium in the fifth and sixth subculture showed virulence for animals would indicate that the virus had been propagated artificially.

This is not considered as establishing definitely that the artificial cultivation of the virus of rabies has been accomplished, but as encouraging evidence that some modification of the methods described may lead to its successful cultivation.

THE RELATION OF THE NITRATES TO THE PUTRESCIBILITY OF SEWAGES.*

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Weldert¹ has rather recently called attention to the treatment of sewages and sludges by the addition of nitrates in the form of saltpeter, and several workers as Guth and Keim,² Bach,³ and Glaser⁴ have since done considerable practical and research work on the same question. The subject is by no means a new one. As far back as 1892 Parry and Adeney⁵ in an article entitled "Purification of Sewage by Microbes" recommended the addition of nitrates to sewage to prevent its becoming putrescent. The practical experiments of the two last named authors extended over four years. The sewage was first strained and treated with manganate of soda before the saltpeter was added. The addition of saltpeter to supply the sewage with the oxygen necessary to make it stable was not offered at that time as a final solution of the problem, but was regarded as very promising in its possibilities. At the time when the article appeared, knowledge of the importance of bacteria and enzymes in the biologic treatment of sewage was already well established. Even previous to these experiments it was known that nitrates would disappear quickly from sewage on standing, particularly when the temperature was favorable.

Scott-Moncrieff⁶ had a process patented in 1898, which consisted in the addition of a highly nitrified filter effluent to septic tank sewages in order to make the sewage more suited for further biologic treatment. Fowler⁷ experimented in 1900 on the purification of septic tank sewage by the addition of filter effluents. All of these experiments seemed to have given results which tho excellent have

* Received for publication July 5, 1913.

¹ *Mitt. kgl. Prüfungsanst. Wasserversorgung*, 1910, 13, p. 96.

² *Ges. Ing.*, 1912, 35, p. 57.

³ *Eng. Record*, 1892, 26, p. 380.

⁴ *Ibid.*, p. 341.

⁵ *English Patent*, No. 4994.

⁶ *Arch. f. Hyg.*, 1913, 80, p. 165.

⁷ *The Manchester Sewage Works* (Suppl.), 1900.

apparently been ignored until recently, when interest in the subject was again renewed.

Weldert speaks very enthusiastically of the possibilities of the application of saltpeter to sewages and sludges and urges sewage experts to try the process under various conditions. The consensus of opinion among recent observers has been that the process is too expensive to be of more than theoretical interest. Anyone interested in the complete bibliography of the process will find a list of references appended in Guth and Keim's, and Glaser's articles.

Weldert finds that the quantity of saltpeter required to make a sewage non-putrescible will vary with the concentration of the sewage and its state of decomposition. Sufficient time must be given for the completion of the reaction, which has been found to be between two and four days. The experiments were made with domestic sewages and sewages containing more or less trade waste. A medium temperature is most suitable. The quantity of Chile saltpeter required to make the sewage stable varied from 834 to 8,340 pounds per million gallons of settled sewage. Guth and Keim worked with stale hospital sewage, fresh domestic sewage, and creamery and brewery trade waste. As far as the trade wastes were concerned the result was unsatisfactory and biologic treatment seemed indispensable. Good results were obtained, however, with the other sewages on the addition of 4,170 to 8,340 pounds per million gallons of sewage, the quantity depending upon the concentration. Each of the Emschergenossenschaft applied quantities of saltpeter, in some cases as high as 25,020 pounds per million gallons of sewage, permitting four days' contact, but on the whole he found 8,340 pounds sufficient. Glaser, the latest observer, found on experimenting with domestic sewage of Vienna that like quantities were required to obtain satisfactory results. When four grains of saltpeter were added to one gallon of sewage, methylene blue still retained its color after three days. A close agreement in the quantities of saltpeter necessary can be noted among those observers. The saltpeter employed in the practical tests was Chile saltpeter, which is crude sodium nitrate of approximately 90 per cent purity, while the laboratory tests were made with the crude and the pure salt.

All of the practical workers agree that the necessary period of contact with the sewage and the cost of the chemical make the treatment impracticable, as simple and successful as it is. Weldert figures the cost of the sewage treatment on the basis of his experiments as varying between \$18 and \$180 per million gallons, and the cost of the sludge treatment as varying between \$0.06 and \$0.32 per cubic yard. Two and one-half to 13.5 pounds of Chile salt-peter were added to one cubic yard of sludge to prevent the formation of hydrogen sulfid, which usually accompanies the decomposition of the sludge.

There is also a fairly close agreement on the physical and chemical changes brought about in sewages by the addition of saltpeter. It is claimed that sewage containing a sufficient amount of saltpeter never develops a putrid odor, that the clearness is increased, and that the organic nitrogen, free ammonia, and "oxygen consumed" are decreased, often, however, but very slightly. Guth and Keim showed that the alkalinity is increased at the end of the reaction due to the combination of the saltpeter sodium and the carbon dioxide.

Opinions on the mechanism of the reaction vary greatly. The question is still an open one, altho it is certain that the presence of microorganisms is essential. Guth and Keim demonstrated that bacterial development is not retarded on the addition of saltpeter and that its action is not that of a preservative. No reduction took place on the addition of saltpeter to sterile sewage. Letts, Blake and Totton¹ give a description of the chemical products formed when saltpeter is added to sewage. The nitrate nitrogen was found in their experiments to be converted into free ammonia and nitric oxide; often only free nitrogen was formed. There is no evidence from those authors' experiments which would show that any considerable quantity of these gases was produced from either the free ammonia or the organic nitrogen present. "The process is one of combustion, the oxygen of the potassium nitrate appearing either partly or entirely in the form of carbon dioxide." In some of the experiments (anaerobic incubation of sewage with saltpeter) a little nitrite was produced, but ordinarily no nitrite could be

¹ *Chem. News*, 1903, 83, p. 182.

detected at the end of the reaction. The formation of free nitrogen on the addition of saltpeter to sewage has also been observed by others. Contrary to Bach and Guth and Keim, Glaser claims that the reduction to free nitrogen takes place directly and that no nitrite is formed during the process.

In the United States, a limited amount of work on the addition of saltpeter to sewage on contact filters was done at the experimental sewage testing station at Columbus, Ohio, during 1904-5.¹ Sodium nitrate was added, in quantities of 20 to 30 parts per million of nitrogen as nitrates, to the contact filters to stimulate nitrification. The sewage was permitted to stand for eight hours. The application did not seem to have any influence upon the oxygen in solution, but a considerable decrease of "oxygen consumed" was noted. The reduction of the applied nitrate nitrogen within the first one or two hours was marked. In a number of subsequent experiments the period of contact with saltpeter was allowed to reach one week. No permanent improvement was noted, but it was observed with interest, that the last portion of the nitrate was reduced very slowly and that the organic nitrogen and "oxygen consumed" were decreased when the period of contact reached 24 to 48 hours. It was thought that the nitrate might supply the difference in oxygen, thus establishing more effective bacterial conditions. In this the experiments have been disappointing.

Some of my experiments given later are somewhat in line with the ones made at Columbus. Undoubtedly there must have been an improvement in the stability of the treated sewage in Columbus, which, however, is not indicated in the report. Unfortunately the addition of saltpeter cannot take the place of free oxygen in the immediate destruction of organic matter, but it will be noted later that it results in the shortening of the ultra-anaerobic period. The nitrate oxygen must be utilized by the bacteria and thus improve the sewage or effluent sooner or later. Anyone expecting immediate benefits from the saltpeter treatment is bound to be disappointed.

The attention of the writer was first drawn to this subject while investigating the rôle played by the nitrate oxygen and the free

¹ G. A. Johnson, *Report on Sewage Purification at Columbus, Ohio*, p. 254.

dissolved oxygen in Spitta and Weldert's methylene blue putrescibility test. The results obtained in this investigation seemed of sufficient interest to be reported. Spitta and Weldert¹ were the first to utilize the decoloration of methylene blue in the absence of oxygen as an index of the degree of the putrescibility of a liquid. This test, which is almost universally used at present, has been investigated in this country in particular by Phelps and Winslow,² and the time of decoloration on anaerobic incubation has been found to coincide with the disappearance of the total available oxygen consisting of the free, nitrate, and nitrite oxygen. These forms of oxygen are given here in the order in which they were found to disappear. Abroad, the time required for decoloration serves as the expression of the degree of putrescibility of a sewage, effluent, or contaminated water, while in this country it is customary to report, as suggested by Phelps,³ the degree of putrescibility of a given sample in terms of "relative stability," which indicates the ratio of the available oxygen to the oxygen required for complete oxidation. It is pretty well established that the appearance of gases, such as hydrogen sulfid, which formerly served as the most reliable index of putrefactive conditions, coincides closely with the formation of the colorless leuko-base of the organic dye.

Since there is no doubt that putrefactive conditions will not set in as long as available oxygen is present in any form, it seemed of interest to look closer into the time element required for the utilization of the nitrate-nitrite oxygen versus the free oxygen by the bacterial flora. The question is one of importance, since the absence of the free oxygen is often looked upon as the sole index of undesirable conditions. The fact is, that at times there may be enough nitrate and nitrite oxygen present to prevent an actual nuisance and that there is a tendency to overlook this point.

My experiments were carried on at first without the artificial addition of mineral nitrogen. Crude domestic sewage, settled and septic sewage, and sprinkling filter effluents from the sewage testing station of the Sanitary District of Chicago were employed, and later the water from the main channel of the Sanitary District of Chicago.

¹ *Mitt. kgl. Prüfungsanst. Wasserversorgung*, 1906, 6, p. 161.

² *Jour. Infect. Dis., Suppl.* 3, 1907, p. 1.

³ *Contrib. from Sanit. Research Lab. and Sewage Exper. Sta.*, 1909, 5, p. 77.

The Winkler method, as fully described in the "Standard Methods" of the American Public Health Association, has been employed for the determination of the dissolved oxygen in these experiments. In some of the later experiments, when nitrates were added, nitrites formed in some cases in sufficient amounts to make the endpoint in the titration a difficult one to observe. The method of Rideal and Burgess,¹ which is the Winkler process modified into a colorimetric method, has given fairly satisfactory results in such cases. In short, the method consists in the comparison of the colors obtained in the routine of the Winkler method after the addition of the acid with standards prepared by adding varying definite quantities of potassium permanganate to bottles containing some potassium iodid in acid solution. Fairly accurate readings can be obtained after a little practice. Care must be taken to keep the standards in the dark when not in use and to renew them frequently. The nitrites are determined separately. One atom of oxygen is allowed for two atoms of nitrous nitrogen and the amount is subtracted from the total colorimetric reading obtained. An accurate titration method is undoubtedly to be preferred to a colorimetric procedure, but where close accuracy is not a *sine qua non* it is a convenient one. The method has been applied in only one set of the experiments, that is in the experiments in which saltpeter was added in open vessels to sewages and effluents in order to observe the reabsorption of oxygen by the liquids. Even then the samples were titrated except for the short periods of high nitrites during the "nitrate digestion" process, if I may be permitted to call it that. A more detailed study of the colorimetric Rideal-Burgess method is being carried on in the laboratory of the Sanitary District at present. The putrescibility tests were carried on for the most part in eight-ounce glass-stoppered bottles and the quantity of methylene blue calculated so as to give 1 c.c. of a 0.05 per cent watery methylene blue solution for each 150 c.c. bottle capacity, which is within the requirements of the "Standard Methods." The methylene blue employed was the double zinc salt furnished by W. Kip, 69 Barclay St., New York City. Incubations were carried on at 20° C. In some of the saltpeter experiments sterile four-ounce bottles with a tightly fitting cork stopper of good quality were utilized for the putrescibility tests. With a good grade of cork stopper there is at 20° C. incubation little danger from reaeration through leakage. As a matter of fact, glass stoppers are often interchanged and since these stoppers rarely fit perfectly in the wrong bottles, reoxygenation is more likely to occur than with the cork-stoppered bottles. The rim of the glass stoppers was covered with vaseline to prevent the absorption of oxygen as an additional precaution.

Samples which had to be stored for nitrate and nitrite examinations on the following day were preserved and put in the refrigerator, but whenever possible the determinations were made immediately.

The first question of moment was to determine whether the nitrates in a sewage, effluent, or contaminated water are drawn upon simultaneously with the free oxygen, or whether the free oxygen is eliminated alone. In a set of tabulated results, received from Mr. C. B. Hoover of Columbus in the latter part of 1912, I noted that in making incubation tests the nitrate-nitrite oxygen became in nearly all cases very low as the free oxygen disappeared and I was interested to learn whether it would work out likewise with the sewages and effluents used by me.

To make the tests, the liquid was collected in a large vessel, strained through cotton in order to get rid of the coarse suspended matter, brought up to room tempera-

¹ *Analyst*, 1909, 34, p. 193.

ture gradually, and siphoned carefully, so as to avoid aeration, into glass-stoppered bottles. The crude sewage as a rule was fresh and contained considerable free oxygen in solution. On filtration through cotton the oxygen was still further increased. The dissolved oxygen, the nitrates and nitrites were determined at the start and at the time when the dissolved oxygen had just disappeared. The nitrites and nitrates were calculated as parts per million of nitrogen. The figures were carried to the first decimal place only, except where greater accuracy seemed desirable. The crude sewage used in these experiments was a fresh domestic sewage from a large sewerage area. A sprinkling filter effluent and a trade waste were used in addition.

The results obtained with crude sewage are given in Table 1.

TABLE 1.

CHANGES IN THE NITRITE AND THE NITRATE CONTENT OF CRUDE SEWAGE AT THE POINT OF FREE OXYGEN EXHAUSTION.

Initial Dissolved Oxygen Parts per Million	Initial Nitrite as N. Parts per Million	Initial Nitrate as N. Parts per Million	Dis-solved Oxygen Exhaust. in Hours	Dis-solved Oxygen at End of Test Parts per Million	Nitrite at End of Test Parts per Million	Nitrate at End of Test Parts per Million	Change in Nitrite	Change in Nitrate	Nitrite Increase Percentage	Nitrate Decrease Percentage
5.0.....	0.08	0.7	5	0.0	0.1	0.5	+0.02	-0.2	25	20
5.3.....	0.06	0.55	6	trace	0.3	0.1	+0.24	-0.45	400	82
5.0.....	0.2	1.5	5½	trace	0.3	1.3	+0.1	-0.2	50	13
4.7.....	0.2	1.5	0.5	0.3	1.3	+0.1	-0.2	50	13
5.0.....	0.15	2.0	7	trace	0.3	1.4	+0.15	-0.6	100	30
5.0.....	0.2	1.7	0.2	0.2	1.4	0.0	-0.3	0	18
5.5.....	0.06	0.7	5	trace	0.2	0.6	+0.14	-0.1	233	14
4.8.....	0.08	0.55	4½	trace	0.08	0.5	0.0	-0.05	0	9
6.3.....	0.04	0.65	5	trace	0.1	0.55	+0.06	-0.1	150	15
5.6.....	0.08	0.85	6	trace	0.2	0.45	+0.12	-0.4	150	47
5.0.....	0.08	0.7	5	trace	0.15	0.35	+0.07	-0.35	88	50
5.6.....	0.08	0.6	5	trace	0.15	0.5	+0.07	-0.1	88	17
Aver. 5.4.....	0.11	1.00	5½	0.06	0.2	0.74	+0.09	-0.26	82	26

We observe uniformly that the nitrites are increased considerably on account of the reduction of the nitrates. Still not all of the nitrate lost at the point of free oxygen exhaustion can be accounted for in the increase of the nitrites, and there is no doubt that part of the nitrates became still further reduced, probably to ammonia and free nitrogen. The increase in nitrites of the average above (0.09 parts per million nitrogen) would only amount to 0.12 nitrate nitrogen (proportion NO_2 to NO_3) which still leaves 0.14 p.p.m. of nitrate nitrogen or 54 per cent unaccounted for. The oxygen available from the nitrites at the start averages 0.25 p.p.m.; from the nitrates 3.43 p.p.m. or in total about 3.7 p.p.m., which is over 68 per cent of the initial free oxygen figure. The total available oxygen was therefore 9.1 p.p.m., of which there is left 0.46 p.p.m.

for the nitrites, 2.53 p.p.m. for the nitrates, which added to the very small quantity of free oxygen left amounts to a residual total available oxygen of about 3.0 p.p.m. The questions of the relation of the residual total available oxygen to the stability of a sewage after the dissolved oxygen has disappeared and whether the rate of absorption of the total available oxygen goes on in a uniform manner are discussed later.

The changes were also observed in the nitrites and nitrates at the point of oxygen exhaustion in sewages mixed with lake water in various proportions (Table 2). The Lake Michigan water is practically negligible as far as its nitrite-nitrate content is concerned. Only three experiments in all were made in this series. In the three mixtures employed there was enough oxygen present to prevent its entire complete loss and the samples were examined for nitrites and nitrates when the low point was reached.

TABLE 2.*

CHANGES IN THE NITRITE AND NITRATE CONTENT OF CRUDE SEWAGE-LAKE WATER MIXTURES AT THE POINT OF FREE OXYGEN EXHAUSTION.

Mixture	Initial Dis- solved Oxygen p.p.m.	Initial Nitrite as N. p.p.m.	Initial Nitrate as N. p.p.m.	Dis- solved Oxygen at End of Test p.p.m.	Nitrite at End of Test p.p.m.	Nitrate at End of Test p.p.m.	Change in Nitrites	Change in Ni- trates	Nitrite Incr. Per- centage	Nitrate Decr. Per- centage
tS + 5W.....	9.8	0.03	0.3	0.5	0.03	0.2	0.0	-0.1	0	33
tS + 6W.....	9.6	0.02	0.3	1.5	0.04	0.2	+0.02	-0.1	50	33
tS + 5W.....	9.7	0.03	0.4	0.3	0.04	0.3	+0.01	-0.1	33	25
Average.....	9.7	0.027	0.33	0.77	0.037	0.23	+0.01	-0.1	37	30

* S indicates sewage; W indicates lake water.

We note again in these three tests an increase in the nitrites and a decrease in the nitrates at a point when the free oxygen is very low. The following calculation is based on the average obtained:

	Beginning of Test	End of Test
Total oxygen available.....	10.9 p.p.m.	1.6 p.p.m.
Oxygen available from nitrites.....	0.06 p.p.m.	0.08 p.p.m.
Oxygen available from nitrates.....	1.1 p.p.m.	0.8 p.p.m.

The result of a number of similar experiments made with mixtures of crude sewage and sprinkling filter effluents in various proportions are given in Table 3. "S" again indicates sewage and "F" filter effluent. It may be mentioned that the filter effluents by themselves are non-putrescible, contain considerable quantities of nitrates and

oxygen in solution, and that the sewage was added for the purpose of making the mixtures putrescible and permitting the elimination of the free oxygen.

TABLE 3.

CHANGES IN THE NITRITE AND NITRATE CONTENT OF CRUDE SEWAGE-FILTER EFFLUENT MIXTURES AT THE POINT OF FREE OXYGEN EXHAUSTION.

Mixture	Initial Dis-solved Oxygen p.p.m.	Initial Nitrite as N. p.p.m.	Initial Nitrate as N. p.p.m.	Dis-solved at End of Test p.p.m.	Nitrite at End of Test p.p.m.	Nitrate at End of Test p.p.m.	Change in Nitrites	Change in Nitrates	Nitrite In-crease Per-centage	Nitrate De-crease Per-centage
1S+7F.....	7.6	0.15	2.0	0.5	0.8	0.01	+0.65	-1.99	433	99.5
2S+7F.....	6.9	0.15	1.8	trace	0.3	0.04	+0.15	-1.76	100	92
1S+7F.....	7.7	0.2	5.0	trace	0.25	4.4	+0.05	-0.6	25	12
1S+1F.....	6.6	0.2	3.3	0.8	1.0	2.2	+0.8	-1.1	400	33
1S+1F.....	6.1	0.15	2.6	0.9	0.55	1.8	+0.4	-0.8	266	31
Aver.	7.0	0.17	2.9	0.44	0.58	1.7	+0.41	-1.2	241	41

From the average the following results are calculated:

	Beginning of Test	End of Test
Total oxygen available.....	17.3 p.p.m.	7.5 p.p.m.
Oxygen available from nitrites.....	0.38 p.p.m.	1.3 p.p.m.
Oxygen available from nitrates.....	9.9 p.p.m.	5.8 p.p.m.

From the average of every set of the experiments it may be noted that the loss from the nitrate-oxygen exceeds the gain from the nitrite-oxygen, so that we have to register a loss in combined nitrate-nitrite oxygen at the point when the dissolved oxygen is lowest. This loss is particularly marked in the highly nitrified sewage-filter effluent mixture, representing approximately 16 per cent of the total available or 40 per cent of the initial free oxygen present. It seems reasonable to assume that the rate of absorption of the free oxygen would be influenced by a high nitrate and nitrite oxygen content.

Since there is no doubt that the mineral-bound oxygen goes to satisfy putrescible organic matter, it is evident that the oxygen in this form must be of great importance under certain conditions. In a sprinkling filter effluent a high nitrate and free oxygen content is ordinarily taken as the index of the efficiency, yet with a high oxygen content and a low nitrate content, representing winter conditions, the effluent may be non-putrescible. Under these conditions it becomes of importance to learn the relation of the nitrate oxygen to the stability of a sewage liquid. For this purpose determinations were made in various sewage mixtures of the initial available oxygen, and the time was compared which it took for the methylene blue to disappear after all of the free oxygen was exhausted. Otherwise the technic of the test remained as previously described. A number of bottles were filled with the sewage, which had been previously brought up to 20° C. at the start, besides filling the putrescibility bottles for frequent dissolved

oxygen determinations in order to be able to determine the nitrites and nitrates at the point of free oxygen exhaustion. For convenience, the results are tabulated separately for the sewages and the sewage mixtures (Tables 4 and 5). The temperature of incubation was 20° C. as before.

TABLE 4.

PROPORTION OF TIME REQUIRED FOR THE DECOLORATION OF METHYLENE BLUE BY THE FREE OXYGEN AND THE NITRITE-NITRATE OXYGEN IN CRUDE SEWAGE.

Initial Free Oxygen p.p.m.	Initial Nitrite-Nitrate Oxygen p.p.m.	Free Oxygen Lost Hrs.	Residual Oxygen p.p.m.	Residual Nitrite-Nitrate Oxygen p.p.m.	Time of Decoloration Hrs.	Relative Stability	Nitrite-Nitrate Oxygen at Time of Decoloration p.p.m.	Quantity of 0.05 Per Cent Methylene Blue Solution per 150 c.c. Capacity in c.c.
7.3.....	1.6	8	0.1	0.3	25	21	0.85
5.9.....	1.8	5	trace	1.2	18	16	0.85
5.2.....	1.7	5.15	0.0	1.3	20	17	0.85
6.1.....	1.2	6	trace	0.8	26	22	0.85
3.7.....	1.5	4.15	0.3	1.2	14	12	0.85
6.3.....	2.4	7	0.4	2.1	29	24	0.2	0.70
5.5.....	3.5	5	trace	2.4	20	17	0.3	1.00
5.9.....	7.0	7	0.0	5.4	41	32	0.3	0.70
5.9.....	6.4	6.30	0.2	5.2	27	22	0.2	0.70
Average 5.8.....	3.0	6	0.1	2.2	24	20	0.25	

Table 4 brings out a number of interesting points. Foremost is the fact that if we accept the methylene blue putrescibility test in its present form as a fairly accurate measure of the stability of a sewage, the influence of the nitrate oxygen (the nitrite-nitrate oxygen in the table above is almost entirely nitrate oxygen) in retarding undesirable anaerobic conditions is of greater weight than is commonly appreciated. The sewages above have been subjected to short aeration, in order to increase the free oxygen present, but this could not have had any influence upon the nitrates since their increase or decrease is the result of bacterial activity. It would be impossible, of course, to state definitely to just what extent a certain quantity of mineral nitrogen would influence the stability since this will depend altogether on the biologic life and the degree of decomposition of a sewage. This, as well as other observations, is given with the object of showing the general broad tendency rather than a definite numerical relation. Therefore when the average result in the table above shows that 25 per cent of the initial total available oxygen (consisting of nitrate oxygen) delayed the appearance of anaerobic conditions approximately 200 per cent as measured by the time element, it does not imply by any means that such figures will

always result. One cannot fail to be impressed, however, with the uniformly decided difference in the time element required for the disappearance of the free oxygen and the nitrite-nitrate oxygen which is altogether in favor of the latter.

My early results, which were of a preliminary character and are not recorded here, were still more marked, in fact so surprising that I was led to look for grave errors in the methylene blue test as applied by me. Some interesting figures relating to this subject are contained in an article written by C. B. Hoover,¹ who compared the methylene blue test with three other stability tests applied to sprinkling filter effluents of the Columbus sewage treatment plant and found the results of the test as apt to be misleading, because an effluent may give a very satisfactory stability when in fact only about one-half as good as indicated by the test.

Comparative tests with four different brands of methylene blue convinced me soon that the discrepancy could not be attributed to the brand which I employed. It further suggested that possibly the quantity of the coloring matter might have an influence. Such an observation has been previously made by Jackson and Horton,² who employed 1 c.c. of a 0.05 per cent solution for each 250 c.c. bottle capacity, while the "Standard Methods" recommends a more concentrated solution, namely 1 c.c. of a 0.05 per cent solution for each 150 to 200 c.c. capacity. The quantity which I have applied in the preliminary tests was 1 c.c. per 150 c.c. capacity, but later results were obtained with considerably lower concentrations.

There is no doubt that different results are obtained with varying quantities of methylene blue. Hence lesser concentrations of the dye come closer to indicating the absence of available oxygen. The concentrations recommended by the "Standard Methods" of the American Public Health Association, therefore, seem too high. The difference is particularly marked in the purer samples, such as filter effluents and waters moderately contaminated. A large number of tests have been carried on in this connection. The matter is still being pursued in this laboratory and will be presented in due time. In crude sewages the point raised is of course of much less weight. I feel safe in stating, however, that while the time of decoloration in

¹ *Eng. News*, 1912, 68, p. 452.

² *Jour. Ind. Eng. Chem.*, 1909, 6, p. 328.

Table 4 would be considerably lower if smaller quantities of blue had been used, the results would still have been such as to give the comparatively small amount of nitrate-oxygen the greater share of the time consumed in the utilization of the initial available oxygen.

Most observers who have worked on the methylene blue test have employed an incubation temperature of 38° C., which is convenient from a practical working standpoint, inasmuch as comparative results can be more quickly obtained. It is evident that since the biologic changes, assuming that they are the same at blood temperature as they are at 20° C., will take place much more rapidly at the higher temperature, differences in the decoloration on account of varying quantities of the dye are less noticeable and therefore more likely to be overlooked. Relatively, however, slight difference at the higher temperature will equal the more marked difference at the lower, thereby resulting in serious errors of interpretation. It would be advisable to investigate the influence which different quantities of methylene blue have upon the time of decoloration, when blood temperature incubation is employed. It is not likely that crude sewages would show a material difference, but fairly stable filter effluents might.

A series of tests, similar to those compiled in Table 4, were made with sewage-filter effluent mixtures, which show the same relation as discussed before. The crude sewage was not aerated previously since the mixtures contain considerable free oxygen. The amount of methylene blue employed was uniformly 0.70 c.c. per 150 c.c. capacity, therefore less than recommended by the "Standard Methods."

TABLE 5.

PROPORTION OF TIME REQUIRED FOR THE DECOLORATION OF METHYLENE BLUE BY THE FREE OXYGEN AND THE NITRITE-NITRATE OXYGEN IN CRUDE SEWAGE-FILTER EFFLUENT MIXTURES.

Mixture	Initial Free Oxygen p.p.m.	Initial Nitrite-Nitrate Oxygen p.p.m.	Free Oxygen Lost Hrs.	Residual Oxygen p.p.m.	Residual Nitrite-Nitrate Oxygen	Time of Decoloration	Relative Stability	Nitrite-Nitrate Oxygen at Time of Decoloration p.p.m.
1S+8F.....	7.7	13.6	24	0.5	8.4	7 days	80	0.5
1S+7F.....	8.3	6.2	22	0.07	5.4	7 days	80	0.2
1S+1F.....	6.1	9.1	10	0.9	7.3	48 hrs.	36	0.3
2S+7F.....	7.6	8.5	19	0.1	6.1	33 hrs.	27	0.2
Average.....	7.4	9.1	19	0.4	6.8	104 hrs.	62	0.3

S indicates sewage; F, filter effluent.

Here again we are struck by the large proportion of the time element taken up by the nitrate oxygen in the decoloration of the methylene blue. In 19 hours 7.4 p.p.m. of free oxygen disappeared, while 6.8 p.p.m. residual nitrite-nitrate oxygen took an additional 85 hours, judged by the putrescibility test. The nitrite oxygen in these samples is so low as to be practically negligible. It amounts to only 0.3 per cent of the initial nitrite-nitrate oxygen. However, on account of the active nitrate reduction taking place as the free oxygen disappears, there is an increase in the quantity of nitrites at the low free oxygen point.

In connection with certain dilution experiments, carried on by me under the direction of Mr. Langdon Pearse of the Sanitary District, with a slaughter-house waste, I have had an opportunity to study the relation of its nitrate content to the time required for the decoloration of the methylene blue. The quantity of blue applied was 0.85 c.c. per 150 c.c. bottle capacity. A question mark after a result indicates that the time of decoloration has been estimated. Only such results as could be estimated with a fair degree of accuracy have been taken into account. The waste is high in organic nitrogen and the nitrates present undoubtedly originate to a large extent from various processes. Unfortunately lack of time prevented me from determining more than the initial nitrites and nitrates present and I have to assume that the nitrates are virtually eliminated at the time of the disappearance of the blue color. Nevertheless, the following table contains sufficient concrete data to be of interest.

TABLE 6.
PROPORTION OF TIME REQUIRED FOR THE DECOLORATION OF METHYLENE BLUE BY THE FREE OXYGEN
AND NITRATE OXYGEN IN A SLAUGHTERING-HOUSE WASTE.

INITIAL		FREE OXYGEN LOST IN HRS. APPROXI- MATELY	TIME OF DE- COLORATION HRS.	RELATIVE STABILITY	PERCENTAGE OF TIME OCCUPIED IN THE METHYLENE BLUE TEST BY THE	
Free Oxygen p.p.m.	Nitrite- Nitrate Oxygen p.p.m.				Free Oxygen	Nitrite- Nitrate Oxygen
4.2.....	13.6	6	14 [?]	12 [?]	43	57
3.5.....	7.3	6 [?]	14 [?]	12 [?]	43	57
1.9.....	10.7	6 [?]	14	12	43	57
4.0.....	7.7	6 [?]	15	13	40	60
2.7.....	7.9	6 [?]	10	9	60	40
2.4.....	10.2	6 [?]	14	12	43	57
Average 3.1.....	10.6	6 [?]	13	12	45	55

The mineral nitrogen, in this case, does not seem to have the relatively great influence upon the putrescibility observed with the sewages and filter effluents. Of the average nitrite-nitrate oxygen

above only 0.9 p.p.m. represents nitrite oxygen. While the nitrite-nitrate oxygen is more than three times larger than the free oxygen, it seems to defer the methylene blue endpoint by only approximately 100 per cent. The free oxygen is, therefore, contrary to the former observations, in this case of greater weight than the nitrite-nitrate oxygen. This being a peculiar trade waste of a highly putrescible character, it cannot be directly compared to a normal sewage or effluent in this respect. If an explanation is called for, however, it is to be searched for in the fact that the amount of nitrate is too small to exert a stabilizing influence upon the waste. The bacterial flora is different from the one in ordinary sewage and the oxygen required is far greater than the oxygen available. It is reasonable to assume that the proportional ratio of exhaustion of the two oxygen components, the free and nitrate oxygen, will vary with the quantity of nitrate present, as well as with the oxygen consuming power of a sewage. We shall later see that the addition of a larger quantity of saltpeter to sewage is without influence upon the rate of free oxygen exhaustion and that the addition of a comparatively small quantity of saltpeter is practically without influence upon the stability.

The observations recorded in the previous tables led up to the experiments on the possible improvement of sewages on the addition of nitrates. A solution was prepared of chemically pure potassium nitrate and its strength calculated so that 1 c.c. in a 100 c.c. bottle would be equivalent to 1 p.p.m. of nitrogen, which in turn is equivalent to 7.21 p.p.m. of potassium nitrate or 3.44 p.p.m. of oxygen. A strong solution, ten times more concentrated, was prepared for experiments with larger quantities of the nitrate so as not to dilute the sample unduly. Quite a number of results were lost because the blue color disappeared during the night. Such samples, on which the time of decoloration could not be estimated fairly accurately, were eliminated altogether. In quite a number of the samples, the time could be estimated sufficiently close for my purposes.

The technic of these experiments was as follows: From a large vessel containing sewage, samples were carefully siphoned off to avoid artificial aeration into bottles containing 1 c.c. of methylene blue per 150 c.c. bottle capacity and varying quantities of the potassium nitrate solution. The bottles were then stoppered, incubated at 20° C. and the time of decoloration noted. Up to 5 p.p.m. of nitrogen were added in the form of potassium nitrate. The same sewage was employed as for the previous experiments, only settled. It was of a dilute character, containing on the average about 140 p.p.m. of suspended matter and 36 p.p.m. of chlorin. The nitrates as nitrogen vary anywhere from 0.05 to 0.20 p.p.m. in the crude sewage and from 2.0 to 5.0 p.p.m. as nitrogen in the filter effluents. The nitrites are very low, approximately 0.1 p.p.m.

as nitrogen in the sewage and 0.3 p.p.m. in the filter effluents. The free oxygen in the crude sewage as a rule was around 1.0 p.p.m.

A number of comparative experiments were made with chemically pure sodium nitrate instead of potassium nitrate and with crude saltpeter, containing 96.6 per cent of pure sodium nitrate. It was readily found that the results are identical as long as the amount of nitrate oxygen remains the same. The results obtained are therefore tabulated without reference to the brand of the chemical. Table 7 shows the results obtained on settled non-aerated sewage from the sewage testing station.

TABLE 7.
INFLUENCE OF SALTPETER UPON THE STABILITY OF SETTLED SEWAGES.

	SALTPETER ADDED IN P.P.M. OF NITROGEN								
	0.0	0.1	0.3	0.7	1.0	2.0	3.0	4.0	5.0
Time of Decoloration of Blue Color in Hours	12	12	12	12	12	16	18	20
	23	24	26	30	32	46	58	63
	12	12	12	12	12	19	20	23
	36	36	36	36	38	48	52	64
	22	22	22	22	24	27	29	34
	12	12	12	12	14	21	23	29
	16	16	18	19	20	22	24	26
	14	14	14	14	16	21	25	30
	26	26	26	26	27	34	49	57
	21	22	24	25	25	29	31	36	39
	14	14	14	14	14	16	19	20	21
	12	12	12	12	14	17	21	22	23
	14	14	16	20	21	23
	14	14	20	22	27	30
	17	17	17	17	18	20	21	25	26
	13	13	13	13	16	18	20	21	24
	15	15	15	17	17	19	21	24	25
	15	15	15	16	17	18	20	23	24
Average.....	17	18	18	18.5	21	24	27	31

Table 8 shows the improvement obtained on the addition of saltpeter to settled sewage more clearly. The average figures of Table 7 serve as the basis.

TABLE 8.
IMPROVEMENT IN STABILITY ON THE ADDITION OF VARYING AMOUNTS OF SALTPETER TO SETTLED SEWAGES.

Salt peter Added in p.p.m. of Nitrogen	Oxygen Equivalent in p.p.m. of Oxygen	Time of Decoloration of Blue Color Hrs.	Relative Stability	Improvement Relative Stability Percentage
0.0.....	0.0	17	15
0.1.....	0.3	18	16	6.5
0.3.....	1.0	18	16	6.5
0.7.....	2.4	18.5	16	6.5
1.0.....	3.4	21	18	20.0
2.0.....	6.9	24	21	40.0
3.0.....	10.3	27	22	46.0
4.0.....	13.8	31	25	66.0

The addition of upward of 2.5 p.p.m. of nitrate oxygen seems to have practically no influence upon the stability, but above that

amount the improvement is gradual and marked. The cost of improving the putrescible character of a sewage such as this, represented in the table above by about 65 per cent, would be around \$5.80 per million gallons, on the basis of crude saltpeter in carload lots at two and three-fourths cents per pound. Two hundred and ten pounds of crude saltpeter would be required per million gallons.

A sufficient storage period should ordinarily be provided, but this would depend entirely upon circumstances. Where it would be merely a question of preventing the development of anaerobic conditions in the stream, into which the sewage is to be discharged some distance below, no storage period at all may be required. On the other hand, the quantity of saltpeter to be applied need not necessarily be large enough to supply all of the oxygen for complete oxidation. Only one-half may suffice. This point would likewise depend upon local conditions and could in many cases be quite readily determined. Some subsequent practical experiments, which are reported later, have shown that no odor developed in sewages treated with a sufficient quantity of saltpeter. The sewage cleared up readily, much more so than the untreated sewage, and the sediment appeared to be of a humus-like character. These observations are in accord with those made abroad.

It is of interest to compare a sewage treated with saltpeter with a biologically treated sewage. The two liquids, altho both nonputrescible, can hardly be compared from the standpoint of actual sewage treatment. In one case we have a highly oxygenated clear liquid, low in organic constituents; on the other hand we have the original sewage with the organic matter still present but non-odorless. The transparency of a treated sewage depends upon the storage period. Probably the treatment with saltpeter should be classed as an emergency measure, which would not replace thorough biologic treatment. If the sewage be settled previous to its treatment with saltpeter, a smaller quantity of saltpeter will suffice and the results will be more uniform. The purer the liquid the less saltpeter is required, as is shown by the putrescibility results in Table 9. The improvement is there indicated in the stability on adding the chemical to sewage-lake water mixtures, to sewage-sprinkling filter effluent mixtures, and a contaminated river water.

In a measure, the addition of saltpeter to sewages can be compared to a high supersaturation with oxygen brought about artificially. From this point of view it is of interest to compare the effect upon the stability of simple aeration, as obtained by water falling over a dam, and the addition of saltpeter (Table 13).

I have subsequently made a number of tests to show the quantity of saltpeter necessary to obtain perfect stability of our plain settled sewage and found that an addition of 12 to 18 p.p.m. of nitrogen in the form of saltpeter, representing 41 to 62 p.p.m. of oxygen would do, except when the sewage happened to be a storm water sewage. This would make the cost for chemicals of treating such a settled sewage from \$17 to \$26 per million gallons of sewage. This is based on the assumption, however, that the sewage will receive no other oxygen than the saltpeter oxygen for 20 days, this representing the length of exposure in the methylene blue bottle. It is plain that this standard is very severe and that in most cases it would probably be ample to accept a four days' decoloration as sufficient, in which case, of course, the cost of the saltpeter would be considerably less.

Approximately 50 per cent of the suspended matter in the sewages employed could be settled. For crude, unsettled sewages more saltpeter would be required to obtain like results. A fairly large number of tests would seem to indicate that for the same dry weather sewage, unsettled, approximately 24 to 30 p.p.m. of nitrogen in the form of saltpeter, representing 82 to 103 p.p.m. of oxygen, is necessary, which would make the cost of the chemical between \$35 to \$43 per million gallons. This again is on the 20 days' stability basis.

This investigation was not originally undertaken to determine the merits of the saltpeter treatment of sewage or to suggest such a method as a substitute for the present methods in vogue. The figures on the cost of chemical are given merely because this phase of the problem has presented itself incidentally to the purely technical investigation of the influence of nitrates upon the stability. It is of interest to compare the approximate costs of chemical for this treatment in the United States and abroad. In the United States, the sewages are by far more dilute than on the continent.

Weldert has stated that approximately 8,300 pounds of Chile saltpeter are required for the treatment of one million gallons of sewage. This would make the cost of the chemical alone about \$230, based on a cost of two and three-fourths cents per pound. It would probably require about 800 pounds at a cost of about \$22 to treat a million gallons of our weak nonsettled sewage. The difference in the cost of the treatment of the continental and our sewage can probably be explained by the difference in the dilution of the two. Many factors are involved in this process, all tending to make any figure subject to great variations. My figures are based purely on the particular sewage employed in these experiments. I cannot emphasize strongly enough that each case represents a different problem. This may alter any of the figures cited above materially one way or the other.

A number of results on the addition of saltpeter to sewage-lake water mixtures have been obtained and are given in Table 9.

TABLE 9.
INFLUENCE OF SALTPETER UPON THE STABILITY OF CRUDE SEWAGE-LAKE WATER MIXTURES.

	SALTPETER ADDED IN P.P.M. OF NITROGEN									PROPORTION OF SEWAGE TO WATER
	0.0	0.1	0.3	0.7	1.0	2.0	3.0	4.0	5.0	
Time of Decoloration of Methylene Blue Color in Hrs.	34	34	34	40	44	49	72	1S+5W
	72	72	76	90	96	216	480*	1S+6W
	60	60	65	77	132	480*	1S+6W
	65	67	120	102	480*	1S+5W
	19	20	21	21	22	26	29	31	35	1S+1W
	20	24	25	26	26	27	1S+1W
	25	30	41	50	60	84	3S+4W
	23	23	27	32	34	38	2S+1W

S indicates sewage; W stands for water.

(*) indicates that the blue color persisted after 20 days.

It is not fair in this case to strike an average since a number of results are missing, and since the samples, which held out longer than 20 days cannot be properly counted in. It is plain that the more dilute mixtures require considerably less saltpeter to make them stable than do the more concentrated mixtures. This, of course, is due to the fact that the dilute mixtures contain less organic matter and more free oxygen in solution.

Since most of the sprinkling filter effluents at my disposal were nonputrescible, I had to add various amounts of sewage in order to be able to observe the effect of the addition of saltpeter upon the stability. In Table 10 are shown the results obtained, F indicating filter effluent.

TABLE 10.
INFLUENCE OF SALTPETER UPON THE STABILITY OF SEWAGE-FILTER EFFLUENT MIXTURES.

	SALTPETER ADDED IN P.P.M. OF NITROGEN								PROPORTION OF SEWAGE TO FILTER EFFLUENT
	0.0	0.1	0.3	0.7	1.0	2.0	3.0	4.0	
Time of Decoloration of Methylene Blue Color in Hrs.	24	21	24	24	27	30	30	39	7S+4F
	89	108	120	144	168	288	384	480*	1S+1F
	192	192	252	324	372	396	480*	1S+8F
	46	49	52	58	61	68	132	180	1S+8F
	28	30	32	44	49	58	62	68	2S+7F
	180	180	192	240	264	480*	1S+7F
	86	96	108	120	156	180	192	1S+2F
								252	

(*) again indicates the persistence of the blue color at the end of 20 days.

Table 11 shows the results obtained with the addition of salt-peter to liquid taken from the main channel of the Sanitary District. The average analysis at the time when the tests were made was approximately as follows:

Total suspended matter	53	p.p.m.
Volatile suspended matter	15	p.p.m.
Fixed suspended matter	38	p.p.m.
Oxygen consumed	15	p.p.m.
Free ammonia as nitrogen	1.0	p.p.m.
Organic nitrogen as nitrogen	1.6	p.p.m.
Nitrites as nitrogen	0.04	p.p.m.
Nitrates as nitrogen	0.5	p.p.m.
Chlorin	22	p.p.m.
Alkalinity (as Ca CO ₃)	132	p.p.m.

TABLE 11.
INFLUENCE OF SALTPETER UPON THE STABILITY OF A CONTAMINATED WATER.

	SALTPETER ADDED IN P.P.M. OF NITROGEN.								DISSOLVED OXYGEN ON SPOT
	0.0	0.1	0.3	0.7	1.0	2.0	3.0	4.0	
Time of De- coloration of Methylene Blue in Days	8	8½	10	11½	20*	8.5
	53 hrs.	86 hrs.	4½	12½	20*	4.3
	8	8	11	17½	20*	5.3
	82 hrs.	4½	7	8	9	20*	2.1
	84 "	5	5½	8	9½	20*	3.8
	48 "	72 hrs.	7½	10	20*	1.8
	44 "	46 "	50 hrs.	54 hrs.	73 hrs.	5½	10	20*	2.0
	33 "	70 "	78 "	5	20*	2.0

(*) indicates that the color persisted after 20 days.

The addition of about 1 p.p.m. of nitrogen in the form of salt-peter makes the unstable canal water sufficiently stable for all practical purposes, but the procedure would be too costly. If a

nuisance is to be attacked by this method of treatment at all it can only be attacked by treating the sewage proper, before it has a chance to deoxygenate the river water to the point of the development of odors.

The question is important as to whether the addition of saltpeter has any influence upon the exhaustion of the free oxygen in solution and upon the period of what Phelps styles "oxygen avidity." It is during the period of "oxygen avidity" when the oxygen is on the negative side of the exhaustion curve that nuisance is likely to set in. For this purpose a number of different sewages have been collected in large battery jars and saltpeter added in quantities equivalent to 5 p.p.m. of nitrogen, representing 17.2 p.p.m. of oxygen or 30.3 p.p.m. of sodium nitrate. A "blank" experiment on the same sewage free from saltpeter was carried on at the same time for the sake of comparison. The nitrites and nitrates are expressed in parts per million of nitrogen. The results of the tests are given in Table 12.

The nitrites invariably increased in the sewage treated with saltpeter and in this respect my observation coincides with those of Bach and Guth and Keim. Glaser, as previously mentioned, denies that nitrites are formed during the reduction process.

The effect on purer liquids, such as badly contaminated river waters, of incubation with saltpeter in open vessels has not been studied. The question is therefore open whether saltpeter has a retarding effect upon the deoxygenation of waters which normally do not lose their entire free oxygen on storage. The indications are that sewages lose their free oxygen, whether a reasonable quantity of saltpeter is added or not. It did not seem necessary to use greater quantities of saltpeter than were employed. A distinct difference was noticeable in the appearance of the sewage treated with saltpeter and the "blank" sewage. The "saltpeter" sewage did not develop a putrid odor and also clarified itself long before untreated sewage did. Incidentally, it may be mentioned—and this perhaps is an important point—that when a sewage treated with saltpeter is discharged into a watercourse, the presence of mineral nitrates will greatly promote the development of plankton, which, stimulated by sunlight, furnishes an abundance of free oxygen immediately available for the oxidation of organic sewage matter.

The striking feature of Table 12 is the comparative rate of reoxygenation of the treated sewage and untreated sewage. The period of "oxygen avidity" is considerably shortened. It does not matter whether the free oxygen is exhausted as quickly in the

nitrate sewage, inasmuch as the sewage is in fact provided with sufficient available oxygen to prevent putrefaction. The addition of 5 p.p.m. of nitrogen in the form of saltpeter is equivalent to the

TABLE 12.
INFLUENCE OF SALTPETER UPON THE OXYGEN EXHAUSTION AND REOXYGENATION OF SEWAGES AND SEWAGE MIXTURES.

MIXTURE	SALTPETER SEWAGE			"BLANK" SEWAGE	INITIAL P.P.M.	
	Time Elapsed Hrs.	Dissolved Oxygen p.p.m.	Oxygen Increase p.p.m.	Dissolved Oxygen p.p.m.	Nitrite	Nitrate
Crude sewage	Start	3.3	3.3	0.2	0.35
	4	2.3	2.3
	8	0.08	0.08
	10	0.0
	14	0.0	0.0
	18	0.4	0.4	0.0
	25	0.6	0.6	0.0
	42	3.4	1.1	2.3
3 Pts. crude + 1 Pt. septic + 1 Pt. settled sewage	66	6.2	2.0	4.2
	Start	2.6	2.5	0.3	0.1
	4	1.8	1.8
	6	0.0	0.0
	25	0.4	0.4	0.0
Septic tank effluent.	66	2.0	1.9	0.1
	Start	0.5	0.5	0.2	0.1
	4	0.0	0.0
	25	2.7	0.6	2.1
Settling tank effluent.	66	9.8	3.0	6.8
	Start	1.7	1.6	0.3	0.6
	6	0.3	0.3
	10	Trace	Trace
	42	6.3	1.5	4.8
Crude sewage	66	10.4	2.2	8.2
	Start	5.3	5.3
	29	Trace	0.0
	70	2.1	2.1	0.0
	94	3.0	3.0	Trace

INCUBATION IN CLOSED BOTTLES WITH 4 P.P.M. OF NITROGEN AS SALTPETER.

5 Pts. sewage + 1 Pt. lake water	Start	8.0	8.0
	19	0.3	0.3
1 Pt. sewage + 7 Pts. filter effluent	Start	8.3	8.3
	2½	7.1	7.1
	21	0.14	0.07
Crude sewage	Start	5.5	5.5
	4	0.6	0.3
	5	Trace	Trace

addition of 17.2 p.p.m. of oxygen in a convenient form. If a sewage be aerated at a temperature of 20° C., at best only about 9 p.p.m. of dissolved oxygen is added. This ordinarily is not sufficient to permit aerobic destruction of the putrescible matter present.

Besides aeration other means are not readily available to supply a sufficient amount of oxygen, unless by dilution with fresh water or by the addition of saltpeter.

In order to avoid a serious misunderstanding when comparing these two means of supplying sufficient oxygen, it is very important to remember that an ample dilution with fresh water, if such can be had, is by far the more preferable procedure, disregarding altogether the question of cost. Free oxygen is immediately available for the oxidation of malodorous decomposition products and stimulation of aerobic bacteria. Odors disappear quickly, not because the intensity is lessened in proportion to the dilution, but because the compounds responsible for the odor are actually oxidized. Moreover, the diluted sewage is a physical improvement over raw sewage. The esthetic feature is of importance, altho subordinate to the question of actual nuisance. In the dilution method we have therefore an immediate means of relief. This is not so with the saltpeter method. This method is a prophylactic measure rather than a curative, if I may express it that way. Where an actual nuisance exists, saltpeter will not relieve it at once. There is no immediate chemical reaction between the saltpeter oxygen and the malodorous decomposition products. The saltpeter oxygen becomes available through bacterial activity and its presence will undoubtedly shorten the period of actual nuisance. This will depend, however, upon the progress of decomposition and the amount of nitrite-nitrate oxygen present at the time when the free oxygen has disappeared. The case is different where the prevention of a nuisance is aimed at. A sewage sufficiently dilute in character, or a strong sewage sufficiently diluted with fresh water, may run in an open channel for a considerable period before a live nuisance will develop. Saltpeter added to such a sewage at the point of origin may prevent a nuisance farther down. The time required and the quantity of saltpeter necessary are capable of close determination in the laboratory.

From the standpoint of fish life, a study of Table 12 shows that the addition of saltpeter would not be of immediate benefit, since the essential free oxygen disappears regardless of the saltpeter. The benefit would be derived from the improved ratio of reoxygenation and the increase in plankton, which serves as fish food.

Tests were made to reach a conclusion on the comparative value from the putrescibility standpoint of simple aeration, like that caused by water running over a dam, and the artificial addition of saltpeter. Sewage was aerated and methylene blue tests made on the liquid before and after aeration. To a series of putrescibility bottles containing the nonaerated sewage, various quantities of saltpeter, representing small amounts of available oxygen, were added and the time of decoloration compared with the time required by the aerated sample. The improvement in the aerated sample must necessarily be ascribed to the increased free oxygen. The dissolved oxygen was determined before and after aeration. The results obtained are given in Table 13.

TABLE 13.
IMPROVEMENT IN THE STABILITY OF CRUDE SEWAGES ON AERATION AND ON THE ADDITION OF
SALTPETER.

	SALTPETER ADDED IN P.P.M. OXYGEN							INITIAL FREE OXYGEN IN P.P.M.	INCREASE OF FREE OXYGEN AFTER AERATION P.P.M.	STABILITY AFTER AERATION HRS.
	0.0	0.34	1.02	2.38	3.44	6.88	10.32			
Time of De-	17	17	17	17	18	20	21	4.3	3.9	18
coloration	15	15	15	17	17	19	21	3.5	3.2	17
Hrs.	15	15	15	16	16.5	18.5	20	3.4	4.5	17.5
	15	15	15	16	16	17	18	3.2	4.1	17
	16	16	16.5	16.5	16.5	16.5	18	2.8	5.4	17
	12	13	14	14	14	16	17	3.0	5.2	16
Average,	15	15	15.4	16	16.3	17.8	19.2	3.4	4.4	17.1

We note in the average of above table an increase of 4.4 p.p.m. of oxygen on aeration, which retards the disappearance of the blue color in the putrescibility test by approximately two hours. To bring about such an improvement requires the addition of saltpeter equivalent to 5 p.p.m. of oxygen or 8.8 p.p.m. of saltpeter. Considering the difficulty of obtaining accurate readings of the methylene blue endpoint, there is a fairly close agreement between the improvement in the stability on aeration and the addition of saltpeter.

The question of making sludges nonputrescible by the addition of saltpeter has not been studied, partly because it appears practically a hopeless task from a financial point of view, and partly because there appears to be no advantage to be gained in such treatment. The drying of the sludge in sewage treatment plants in the United States is accomplished without nuisance. As a general proposition, it is much cheaper to have the air and the sunlight mineralize the sludge, even tho it takes longer.

The writer is now studying the possibility of utilizing the addi-

tion of nitrate in laboratory tests to indicate the quantity of oxygen required by sewages, effluents, or streams for complete oxidation. Such information is now obtained by various methods involving dilutions with fresh water. Either the rate of exhaustion of the free oxygen is observed or the dilution is found which is necessary to prevent the decoloration of the methylene blue. Anyone who has tried such dilution tests realizes that the procedure of making dilutions with fresh water is tedious, requiring very great care to avoid aeration. These difficulties become still more pronounced when such tests are attempted in the field. If correct results can be obtained by merely adding to putrescibility bottles different amounts of saltpeter, representing definite equivalents of oxygen, then incubating the bottles at 38° or 22° C., according to time in which the information is desired, the field tests would be very simple. In order to obtain approximate information on the quantity of oxygen needed to complete the oxidation, a blank putrescibility test could be added, and by comparison with the other putrescibility bottles, the information would be gained by noting the smallest quantity of "nitrate" oxygen which will prevent decoloration. If the total quantity of oxygen required is desired, the initial free oxygen, the nitrites, and the nitrates, should be also determined. With incubation at 37° C., results sufficiently close for all practical purposes could be obtained in four days. As a rule, such information is not wanted so urgently that a few days' delay would constitute a serious drawback. The writer is working on such tests and hopes to have material to present later.

The experiments described deal with the direct influence of the nitrates upon the stability. However, since nitrites are formed during the process of nitrate digestion it may be of interest to comment briefly on the relation of nitrite oxygen to the stability.

Nitrites were added artificially in the form of chemically pure sodium nitrite to samples of sewages, sewage-filter effluent mixtures, and polluted water in quantities of 0.2, 0.5, 1.0, 3.0, and 5.0 p.p.m. of nitrogen. The oxygen equivalents of these quantities vary from 0.45 to 11.5 p.p.m. Less oxygen is of course available from the nitrite than from a corresponding amount of nitrate. The oxygen must ultimately be utilized for the oxidation of organic matter. In

most cases, and in particular with the purer samples, the addition of nitrites resulted in a corresponding improvement of the putrescibility as indicated by the methylene blue test. In a number of crude sewages, however, the addition of even 5 p.p.m. of nitrogen as nitrite showed but little if any improvement in the stability. The striking feature observed when nitrites are added to the putrescibility bottles containing crude sewages is the tendency to the reabsorption of atmospheric oxygen. The catalytic action of the nitrites or of the products resulting from their decomposition is pronounced. Manipulation must be very carefully conducted, or the results obtained may be entirely misleading on account of the great affinity of such a liquid for atmospheric oxygen. Frequently a nearly decolorized putrescibility bottle containing sewage has been found to reabsorb oxygen and turn blue inside of a few hours, when the stopper was intentionally slightly loosened, while putrescibility bottles containing decolorized sewage may be loosely stoppered or even contain an air bubble without absorbing the oxygen throughout. Unless the bottle is shaken the reaeration will show only on the surface. The necessity is therefore apparent for employing seals for putrescibility bottles, even when an incubation temperature of 20° C. is used, when effluents are studied which are high nitrites or which form high nitrites on decomposition. Hoover¹ comments on the fact that the methylene blue test is misleading in that the consumption of oxygen in a mixture of water and sprinkling filter effluent is more rapid than in the undiluted sample. His observations are based on incubation at blood temperature, which may not be strictly comparable to observations at 20° C. To a certain extent, it is likely that the phenomenon was due to the oxygen-retaining property of the nitrites, a property which correspondingly weakened in the diluted sample. The fact that the nitrites are the last oxygen-carrying compounds to disappear before anaerobic conditions set in, is in this respect significant. While we know but little of the mechanism of the nitrate reduction as taking place in the decomposition of sewages we have yet to learn a great deal about the rôle which the nitrites play in this process. The physical chemist is the one likely to find an answer to the question.

¹ *Loc. cit.*

SUMMARY.

1. At times there may be enough nitrate-oxygen and nitrite-oxygen present in sewages to prevent ultra-anaerobic conditions. There is a tendency to overlook this point. The influence of oxygen from that source is of greater weight in preventing a nuisance than is commonly appreciated.

2. On incubating sewage mixtures under exclusion of air up to the point of free oxygen exhaustion the nitrites are increased, the nitrates decreased. The loss from the nitrate-oxygen exceeds the gain from the nitrite-oxygen.

3. The concentrations of the methylene blue solution as recommended in the "Standard Methods of Water Analysis" by the American Public Health Association seem too high. This is being investigated further in this laboratory and will be presented at the 1913 annual meeting of the American Public Health Association.

4. Experiments on the addition of saltpeter to the weak domestic sewage of the 39th Street sewerage area in Chicago have demonstrated that it requires approximately 24 to 30 p.p.m. of nitrogen in the form of saltpeter, representing 82 to 103 p.p.m. of oxygen, to obtain complete stability. The cost of the chemical would vary between \$35 to \$43 per million gallons. Settled sewage would require 12 to 18 p.p.m. of nitrogen in the form of saltpeter, representing 41 to 62 p.p.m. of oxygen. This would make the cost of treating the settled sewage from \$17 to \$26 per million gallons.

5. The cost of treating a weak American sewage with saltpeter is much less than the cost of treating a German sewage, probably on account of the higher dilution.

6. The addition of a moderate quantity of saltpeter (equivalent 5 p.p.m. of nitrogen) is without influence upon the rate of free oxygen exhaustion but shortens the period of oxygen "avidity." The addition of a comparatively small quantity of saltpeter (under the equivalent 1 p.p.m. of nitrogen) seems to be practically without influence upon the stability of sewages. The nitrites are invariably increased by the addition of saltpeter to sewage.

7. The addition of 1.0 p.p.m. of nitrogen in the form of saltpeter makes the putrescible waters of the drainage canal of Chicago

stable for all practical purposes, but the procedure is too costly to be of more than theoretical interest.

8. Sewage treated with sufficient saltpeter does not develop a putrid odor and clarifies itself long before an untreated sewage will.

9. The dilution of sewage with fresh water is greatly preferable to the addition of saltpeter, disregarding altogether the question of cost. The dilution method furnishes an immediate means of relief which is not the case with the saltpeter method, in which the sewage may have to be stored for varying periods in order to obtain a fair degree of stability. To fish life the saltpeter treatment is of no immediate benefit for the reason that the essential free oxygen disappears regardless of the presence of the chemical.

10. Experiments have demonstrated that the oxygen obtained by aeration exerts the same effect upon the stability of sewages or contaminated waters as an equivalent quantity of saltpeter-oxygen.

11. The addition of saltpeter may be utilized in connection with the methylene blue putrescibility test to obtain information on the amount of oxygen necessary to complete the oxidation of a sewage or effluent. This method is now being tested in the laboratory of the Sanitary District of Chicago.

12. The addition of nitrites also improves the stability of sewage mixtures. Such a treated sewage has a strong tendency to absorb atmospheric oxygen.

In closing, the writer wishes to acknowledge his indebtedness to Mr. Langdon Pearse, engineer in charge of Sewage Disposal Investigations, for his continued interest in the progress of this work, and to Messrs. Frank Bachmann, A. F. Mellen, A. Dechman, and J. T. Meckstroth for valuable analytical assistance and timely suggestions.

BRILLIANT GREEN BROTH AS A SPECIFIC ENRICHMENT MEDIUM FOR THE PARATYPHOID-ENTERITIDIS GROUP OF BACTERIA.*

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Altho malachite green as an ingredient of solid medium was introduced by Loeffler primarily as an aid in the isolation of the typhoid bacillus, subsequent investigations have demonstrated the greater selective action of this dye for bacilli of the paratyphoid-enteritidis type. Loeffler¹ in 1907 described a nutrose-peptone-lactose-malachite green agar medium which exerts a marked inhibitory action on the development of bacilli of the colon type, but is favorable for the growth of paratyphoid bacilli. This medium, apparently, has given good results in Germany, but as it is rather complicated and troublesome to prepare it does not seem to have been widely adopted. In the following pages is described a very simple and inexpensive fluid medium which may be prepared readily in almost any laboratory and yet which, according to our experiments, exhibits a higher degree of selective availability for growth and isolation of the paratyphoid-enteritidis group than does Loeffler's malachite green solid medium.

Malachite green and brilliant green are both basic dyes and, according to Arthur G. Green,² are closely related. In 1908 Conrad³ suggested the use of brilliant green instead of malachite green as one of the ingredients of a solid medium for the typhoid bacillus, primarily for the reason that brilliant green does not interfere with typhoid agglutination tests. According to the writer's experience neither of these dyes affects the agglutinability of bacilli of the paratyphoid group, and so far as that test is concerned it is a

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¹ *Deutsch. med. Wchnschr.*, 1907, 33, p. 1581.

² *Organic Colouring Matters*, London, 1908. The nature of these two dyes is described under the heading "Triphenylmethane and Diphenylnaphthylmethane Colouring Matters," p. 180.

³ *Centralbl. f. Bakteriöl., Referate*, 1908, 42, Beiheft 1, p. 47.

matter of indifference which dye is used, but in the inhibition of the growth of the colon group and other typically fecal bacteria, brilliant green¹ is more potent than is malachite green and yet it is rather more favorable for the multiplication of bacilli of the paratyphoid-enteritidis group. In the experiment detailed in Table 1, the specified amounts of one per cent aqueous solutions of the dyes (Grübler's) mentioned were added to 10 c.c. of glucose

TABLE 1.
COMPARATIVE TEST OF MALACHITE GREEN (HÖCHST 120) AND BRILLIANT GREEN.

Tube	Dye	Amount of 1 Per Cent Aqueous Solution of Dye Added to 10 c.c. Glucose Broth. c.c.	Cultures Seeded	Number of Bacilli Seeded per 0.5 c.c.	Bacilli per 0.1 c.c. after 48 Hours' Incubation
1.....	Brilliant green	0.15	B. coli mixture	7,000	0
2.....	Malachite "	0.15	" " "	"	0
3.....	" "	0.3	" " "	"	23,000
4.....	" "	0.6	" " "	"	0
5.....	" "	1.0	" " "	"	0
6.....	Brilliant "	0.15	B. paratyphosus B	6	0
7.....	" "	0.3	" " "	"	0
8.....	" "	0.6	" " "	"	0
9.....	Malachite "	0.15	" " "	"	0
10.....	" "	0.3	" " "	"	0
11.....	" "	0.6	" " "	"	0
12.....	" "	1.0	" " "	"	0
13.....	" "	2.0	" " "	"	0
14.....	Brilliant "	0.15	B. enteritidis	6	0
15.....	" "	0.3	" " "	"	0
16.....	" "	0.6	" " "	"	0
17.....	Malachite "	0.15	" " "	"	0
18.....	" "	0.3	" " "	"	0
19.....	" "	0.6	" " "	"	0
20.....	" "	1.0	" " "	"	100,000
21.....	" "	2.0	" " "	"	200

broth, neutral to phenolphthalein. A comparison of Tubes 1-4 shows that 0.15 c.c. of this brilliant green solution (a final dilution of 1-6,600) killed a fairly heavy seeding of a mixture of bacilli of the colon group consisting of *B. coli communis*, *B. coli communior*, *B. acidi lactici*, and *B. lactis aërogenes*, but with malachite green (Höchst 120) an addition of 0.3 to 0.6 c.c. of a one per cent aqueous solution was required to effect the same result. Further, 0.6 c.c. of malachite green which was bactericidal for the colon mixture (Tube 4) also destroyed *B. paratyphosus* B (Tube 11), whereas the

¹ As far as I am aware brilliant green has not been suggested heretofore as an ingredient of an enrichment medium for the paratyphoid group. In 1908 Peabody and Pratt (*Boston Med. and Surg. Jour.*, 1908, 158, p. 213) described a malachite green enrichment-broth medium for the typhoid bacillus, but there are few data available in regard to its efficiency.

same amount of brilliant green checked in no degree the multiplication of this paratyphoid bacillus nor that of *B. enteritidis*. The irregular results in Tubes 11, 12, and 13 were probably due to the fact that the malachite green when added to the broth menstruum in the specified amounts underwent more or less precipitation. These experiments as a whole indicate the superiority of brilliant green over malachite green as an ingredient in an enrichment medium for the paratyphoid-enteritidis group.

After a series of tests of different degrees of acidity of broth combined with varying strengths of brilliant green, the following combination was found to give the optimum differential results. Meat-peptone-broth, prepared according to the usual methods, is titrated to the neutral point for phenolphthalein and one per cent glucose is added. The medium is tubed in exactly 10 c.c. amounts and sterilized in the Arnold. The final reaction must be close to neutral. A one per cent solution of brilliant green (Grüb-ler's) in distilled water is next prepared. This does not require sterilization. Just before the medium is to be used, 0.15 c.c. of this brilliant green solution is added to each tube. This constitutes a final dilution of approximately 1-6,600, which, as has been stated, is a strength sufficient to inhibit or destroy the dominant fecal bacteria and yet exercises no repressive action on members of the paratyphoid-enteritidis group. With the given amount of brilliant green a slight increase in the acidity of the broth causes a marked increase in the bactericidal properties of the medium. At 0.5 acid to phenolphthalein only certain strains of the paratyphoid and enteritidis bacillus would grow, while at 1.0 acid none of a number of strains of this group grew.

An enrichment medium, made according to the methods described above, has been tested with a number of representatives of the paratyphoid-enteritidis group and with other bacteria which occur constantly or occasionally in the intestinal tract. These cultures have been received from a number of different sources and have been adequately identified. All of the cultures¹ placed in the

¹ For cultures numbered 5, 9, 10, 15, 16, 17, 30, 32, and 33 in Table 2, I gratefully acknowledge my indebtedness to Professor C.-E. A. Winslow, Department of Public Health, American Museum of Natural History; and for cultures 3, 4, 6, and 7, to Dr. Krumwiede, Health Department Research Laboratory, New York City.

left-hand column belong definitely in the paratyphoid-enteritidis group, except No. 18 which is a bacillus isolated from dog feces and similar to *B. enteritidis* except that it acts slowly on lactose and is not pathogenic when fed to mice. All of these cultures belonging in the "intermediate group" grew readily in this brilliant green broth except Schottmüller No. 8. This strain had apparently become atypical through conditions of cultivation, because Schottmüller No. 7, which is undoubtedly its original parent strain, multiplied actively in this medium. Of other bacteria, selected as controls, the only species which grew readily in this medium was *B. pyocyaneus*. For other cultures the dye proved either inhibitory or rapidly bactericidal. The pyogenic cocci, *B. dysenteriae*, *B. alkaligenes*, Morgan's bacillus No. 1, and certain strains of *B. coli*, were killed within a few minutes in this medium. This strength of brilliant green also proved toxic for the typhoid bacillus. Certain strains belonging in the colon group, especially *B. coli communior* and *B. acidi lactici*, might exhibit a moderate increase after two to four hours' incubation, but after 24 hours were found to have practically died out. The glucose was added to the medium with the idea that organisms only moderately susceptible to the strength of dye, at the neutral point, by splitting this sugar might so increase the acidity of the medium as to cause their own destruction, for a slight increase in acidity greatly enhances the toxicity of brilliant green. Of the members of the colon group *B. lactis aërogenes* shows the greatest degree of tolerance for this dye, but our experiments have shown that *B. enteritidis* is able to overgrow completely and eliminate this bacillus even when the latter is present originally in much smaller numbers. It may be observed that the seeding of the control cultures was made in general much heavier than that of the paratyphoid-enteritidis strains.

The selective action of brilliant green on groups of bacteria has not been studied as extensively as has that of gentian violet by Churchman.¹ It would seem, however, that brilliant green exerts the finer degree of selective action. Relatively few bacteria have been found with a tolerance for this stain and they were almost exclusively confined to the paratyphoid-enteritidis group. It is

¹ *Jour. Exper. Med.*, 1912, 16, pp. 221 and 822.

possible that other members of the large mucosus capsulatus group may be able to multiply readily in this medium, but as the matter was not pertinent to the purpose of this investigation no tests were made.

TABLE 2.
SHOWING THE SELECTIVE ACTION OF BRILLIANT GREEN GLUCOSE BROTH FOR BACILLI OF THE
PARATYPHOID-ENTERITIDIS GROUP.

Cultures	Plated at Once to Determine Number of Bac- teria Seeded. Result per 0.1 c.c.	Plated after 24 Hours' In- cubation at 37° C. Result per 0.1 c.c.	Cultures	Plated at Once. Bacteria in 0.1 c.c.	Plated after 24 Hours' Incuba- tion
1. <i>B. paratyphosus</i> A (7) . . .	150	∞	19. <i>B. coli communis</i> . . .	1,600	0
2. " " " (116) . . .	975	∞	20. " " " . . .	8,320	520
3. " " " (Y) . . .	1,430	∞	21. " " " communior . . .	7,800	1
4. (Seeman)	250	∞	22. " " " . . .	8,800	100
5. " " " B (22) . . .	485	∞	23. <i>B. acidi lactici</i>	9,900	14
6. " " " (Y) . . .	1,500	∞	24. " " "	11,500	80
7. (Schottmüller)	300	∞	25. " " "	2,145	0
8. <i>B. paratyphosus</i> B (Schottmüller)	2,275	3,900	26. <i>B. lactis aërogenes</i> . . .	15,500	40,000
9. <i>B. enteritidis</i> (132)	2,340	∞	27. <i>B. typhosus</i> (79)	1,560	1
10. " " " (18)	910	∞	28. <i>B. dysenteriae</i> (Kruze) . . .	13,000	20,000
11. " " " (Dog 69)	1,300	∞	29. <i>B. typhosus</i> (79)	8,500	10
12. " " " (Dog 59)	2,470	∞	30. <i>B. dysenteriae</i> (Flexner) . . .	6,000	0
13. " " " (Dog 40)	975	∞	31. Morgan's bacillus No. 1 . . .	19,000	35
14. " " " (Rat)	3,250	∞	32. <i>B. alkaligenes</i> (439) . . .	16,000	0
15. <i>B. suipestifer</i>	1,820	∞	33. <i>B. proteus vulgaris</i> 1 . . .	3,000	0
16. <i>B. typhi murium</i>	1,300	∞	34. " " " 2	6,000	70
17. <i>B. danysz</i>	2,200	∞	35. " " "	4,000	900
18. <i>Bacillus</i> No. 125	910	∞	36. <i>B. pyocyaneus</i>	9,500	∞
			37. <i>Aur. aureus</i>	9,000	0
			38. <i>Alb. pyogenes</i>	9,500	0
			39. <i>Str. pyogenes</i>	2,000	0

In determining the proper strength of brilliant green for this medium, an attempt was made to select that dosage which would not inhibit in any degree the growth of the paratyphoid-enteritidis group, but yet would exert a strong repressive action on the commonly occurring fecal bacteria. The experiment detailed in Table 3 shows that the strength of the dye adopted did not inhibit the development of a typical culture of *B. enteritidis* nor of *B. paratyphosus* B, even when the total seeding amounted to only 4 or 5 bacteria, growth occurring as readily in the brilliant green broth as in the same broth without the dye. For two strains of *B. paratyphosus* A this also held true, but one strain, 116, proved more sensitive to this stain as there occurred no growth even when the total seeding amounted to over 600 bacilli. With a heavier seeding, however, this strain will also grow readily (Table 2, No. 2).

As a rule in an infected stool the paratyphoid bacilli are greatly outnumbered by the normal fecal flora and it is evident that a selective fluid medium to be of value must be so constituted that bacilli of the paratyphoid group may overgrow relatively large

TABLE 3.

SEEDING OF SMALL NUMBERS OF *B. paratyphosus*, TYPES A AND B, INTO BRILLIANT GREEN GLUCOSE BROTH AND INTO GLUCOSE BROTH TO DETERMINE THE COMPARATIVE TENDENCY TO GROWTH.

Dilution Tube	Organism Seeded	Total Number of Bacilli Seeded	Brilliant Green Glucose Broth Tubes after 24 Hours' Incubation	Glucose Broth Tubes after 24 Hours' Incubation
5.....	<i>B. paratyphosus</i> , Type A	84	Growth, dark green, gas.	Growth
6.....	<i>B. paratyphosus</i> , Type A	3	No growth	"
7.....	<i>B. paratyphosus</i> , Type A	0	" "	No growth
5.....	<i>B. paratyphosus</i> Type A (7)	230	Growth	Growth
6.....	<i>B. paratyphosus</i> Type A (7)	11	"	"
7.....	<i>B. paratyphosus</i> Type A (7)	2	"	"
4.....	<i>B. paratyphosus</i> Type A (116)	620	No growth	Growth
5.....	<i>B. paratyphosus</i> Type A (116)	29	" "	"
6.....	<i>B. paratyphosus</i> Type A (116)	6	" "	No growth
5.....	<i>B. enteritidis</i> No. 18	80	Growth	Growth
6.....	" " " "	4	"	"
7.....	" " " "	0	No growth	No growth
5.....	<i>B. paratyphosus</i> (Schottmüller)	90	Growth	Growth
6.....	<i>B. paratyphosus</i> (Schottmüller)	5	"	"
7.....	<i>B. paratyphosus</i> (Schottmüller)	0	No growth	No growth

numbers of these fecal bacteria. The following experiment (Table 4) was designed to determine to what extent this medium is effective as regards the colon group. The colon seeding consisted of a mixture of cultures of *B. coli communis*, *B. coli communior*, *B. lactis aërogenes*, and *B. acidi lactici*. It was found that all of the three representatives of the paratyphoid-enteritidis group when present in the ratio of one paratyphoid to 250 or 300 colon bacilli readily overgrew them to the extent that after 24 hours' incubation pure cultures were present in the tubes. In a ratio of one paratyphoid bacillus up to 180,000 colon bacilli this in large measure still held true, whereas when the colon seeding amounted to 1,800,000 bacilli to one *B. enteritidis*, the latter apparently was unable to multiply.

In view of the fact that malachite green agar is generally recognized as a favorable medium for the isolation of bacilli of the paratyphoid group, any substitute offered may reasonably be expected to equal or surpass its efficiency as a selective medium for the isolation of the paratyphoid group, aside from any other advantages which such a substitute may offer in the way of greater simplicity in preparation. Accordingly comparative tests have been undertaken in which the same samples of infected feces were cultivated on Loeffler's malachite green-nutrose-lactose agar, especially devised by the originator for paratyphoid work, and in the brilliant green glucose broth.

TABLE 4.
COMBINED SEEDING OF *B. coli* MIXTURE AND *B. enteritidis* OR *B. paratyphosus* IN BRILLIANT GREEN
GLUCOSE BROTH.

TOTAL SEEDING		TUBES INCUBATED 24 HOURS AND PLATED ON ENDO MEDIUM
<i>B. coli</i> Mixture	<i>B. enteritidis</i> or <i>B. paratyphosus</i>	
920,000	<i>B. enteritidis</i> 35,000	Pure culture, <i>B. enteritidis</i>
"	" " 6,000	" " " "
"	" paratyphosus B 8,000	" " " <i>B. paratyphosus</i>
"	" " 3,200	" " " "
"	" " (Seeman), 17,500	" " " "
"	" " 6,000	" " " "
28,000,000	" enteritidis 3,900	Nearly pure culture, <i>B. enteritidis</i>
"	" " 360	" " " "
"	" " 160	Few <i>B. coli</i> , no " "
"	" " 120	Nearly pure culture " "
285,000,000	" " 3,900	<i>B. coli</i> reduced, no " "
"	" " 360	" " " "
Control 920,000	None	No growth
" 28,000,000	"	<i>B. coli</i> , 160,000 per 0.1 c.c.
" 285,000,000	"	" 1,000,000 " " "

For the purpose of these comparative tests dogs were fed with boiled milk seeded with 24-hour agar or broth cultures of various strains of the paratyphoid-enteritidis group. It may be noted in passing that the feeding seemed to cause no definite ill effect to the subjects. Generally a transitory diarrhea occurred lasting a day or two, but no other indication of toxic action. This result agrees with the well known natural immunity of dogs to this group of bacteria.

At daily intervals the stools of these subjects were cultivated on the Loeffler malachite green agar and also in the brilliant green glucose broth. Two or three plates of the Loeffler medium were seeded from each specimen of feces. In addition to seeding from

thick emulsions of the feces, a plate was generally streaked with a loop from the undiluted stool. Success attended the second mode of seeding more often than the first. In cultivating the feces with the brilliant green glucose broth the following method was found to give the best results; in fact any deviation from it was likely to be attended with failure.

Method for use of medium in cultivating feces.—To six tubes of 10 c.c. each of glucose broth, neutral to phenolphthalein, 0.15 c.c. of a one per cent aqueous solution of brilliant green is added. It is advisable to add the dye to each tube of broth just before it is to be used as after the lapse of a day or two a slight amount of precipitation will have occurred with a consequent weakening of the medium. In one of these tubes of brilliant green broth from one-fourth to one-half a gram of feces is thoroughly emulsified. This is allowed to settle for about 10 minutes, then 1 c.c. of this emulsion is seeded into one tube of brilliant green broth, 0.5 c.c. into each of two tubes, and three drops into each of two tubes. If the paratyphoid or enteritidis bacilli are fairly numerous in the feces, pure or nearly pure cultures will develop in all of these tubes. If, however, they are present in very small numbers, the specific growth occurs rather more often in the lightly than in the heavily seeded tubes. If *B. lactis aërogenes* is plentiful in the feces it may multiply, but apparently may be overgrown by *B. paratyphosus* B or *B. enteritidis*, even tho at the start the latter are present in the great minority.

The tubes, with the exception of the primary emulsion which is discarded, are incubated for 24 hours at 37° C. Growth is generally indicated by gas formation and a darkening in the shade of green. Streak cultures are then made on Endo plates to determine the type of growth and typical colonies are isolated and subjected to the cultural and agglutination tests necessary for identification. As paratyphoid or enteritidis bacilli when present at all in the incubated tubes are generally in pure or nearly pure culture, early information may be gained by adding a drop of strong specific agglutinating serum to the tube. This test obviously cannot take the place of isolation and proper identification.

In Table 5 are assembled the data in regard to the practical comparative tests of the efficiency of these two media. In the total 18 experiments of this character the bacillus fed to the dogs was recovered, using the Loeffler malachite green plates, in nine instances or 50 per cent, but with the brilliant green broth in 14 instances or 77.7 per cent. Furthermore in five experiments the bacillus fed was isolated with the enrichment medium where it could not be recovered from the Loeffler plates, whereas in no instance was the bacillus recovered with the plate medium where it was not found growing in the brilliant green broth. Aside from its greater efficiency the simplicity of this brilliant green enrichment medium is

much in its favor. In any bacteriological laboratory it could be prepared within an hour or two for an emergency.

TABLE 5.

COMPARATIVE RESULTS IN CULTIVATING THE FECES OF DOGS FED WITH *B. paratyphosus* OR WITH *B. enteritidis* ON LOEFFLER'S MALACHITE GREEN AGAR AND IN BRILLIANT GREEN GLUCOSE BROTH.

Dog	Dose	Examination of Feces after Feeding (Days)	Endo Plates	Loeffler's Malachite Green Plates	Brilliant Green Broth
138.....	<i>B. enteritidis</i> ; 3 broth cultures	4	None made	Negative	Positive; pure culture <i>B. enteritidis</i> in 2 tubes.
139.....	<i>B. enteritidis</i> ; 3 agar cultures	2	Negative	Positive; 4 colonies on 2 plates	Positive; pure culture in all 4 tubes.
139.....	<i>B. enteritidis</i> ; 3 agar cultures	3	"	Positive; 2 colonies	Positive; pure culture <i>B. enteritidis</i> in all 4 tubes.
139.....	<i>B. enteritidis</i> ; 3 agar cultures	4	"	Positive; 1 colony	Positive; pure culture in 1 tube, mixed in others.
140.....	<i>B. enteritidis</i> ; 1 broth culture	1	Negative	Positive; many colonies	Positive; pure culture in 2 tubes.
140.....	<i>B. enteritidis</i> ; 1 broth culture	2	"	Positive; fairly numerous	Positive; almost pure in 4 tubes.
140.....	<i>B. enteritidis</i> ; 1 broth culture	3	"	Positive; 2 colonies	Positive; pure culture in 1 tube.
140.....	<i>B. enteritidis</i> ; 1 broth culture	4	"	Negative	Positive; pure culture in 1 tube.
142.....	<i>B. enteritidis</i> ; 3 agar cultures	3	"	Positive; 2 colonies	Positive; pure in 1 tube.
142.....	<i>B. enteritidis</i> ; 3 agar cultures	4	"	Negative	Positive; almost pure in 1 tube.
142.....	<i>B. enteritidis</i> ; 3 agar cultures	5	"	"	Negative
142.....	<i>B. enteritidis</i> ; 3 agar cultures	6	"	"	"
142.....	<i>B. enteritidis</i> ; 3 agar cultures	7	"	"	Positive; almost pure in 2 tubes.
142.....	<i>B. enteritidis</i> ; 3 agar cultures	10	"	"	Negative
143.....	<i>B. paratyphosus</i> B; 3 agar cultures	4	Negative	Positive; 1 colony	Positive; pure in 1 tube; mixed in 1 tube.
143.....	<i>B. paratyphosus</i> B; 3 agar cultures	5	"	Positive; numerous colonies	Positive; mixed in 2 tubes.
143.....	<i>B. paratyphosus</i> B; 3 agar cultures	6	"	Negative	Negative
143.....	<i>B. paratyphosus</i> B; 3 agar cultures	7	"	"	Positive; almost pure in 2 tubes.
144.....	<i>B. paratyphosus</i> A; $\frac{1}{2}$ broth culture	1	"	Positive; pure cultures.

Both *B. enteritidis* and *B. paratyphosus* B were isolated as late as the seventh day after feeding. In the first or second stool passed after infection by feeding (about 24 hours later) the number of colonies of the bacillus fed might equal on the Endo plates the total number of colonies of the colon type. After 48 hours, however, the bacilli fed had decreased to such a degree that none of their colonies appeared on the Endo plates and after four days they were evidently present in exceedingly small numbers. It is appar-

ent that the intestinal tract of the dog under normal conditions does not offer a favorable environment for bacilli of this type. *B. paratyphosus* A is evidently eliminated from the fecal flora of the dog more quickly than the B type or *B. enteritidis* as it has not been isolated later than 24 hours after feeding.

It is quite possible that this brilliant green enrichment medium may prove of value for investigations in regard to the presence of bacilli of the paratyphoid-enteritidis type in foods suspected of having caused poisoning or in water and ice subject to pollution. In material in which *B. lactis aërogenes* is present in great numbers, as in the poorer grades of milk, this medium should be used with caution because of the danger of overgrowth. In systematic investigations of this character this enrichment medium might be advantageously combined with the Loeffler malachite green agar plates.

SUMMARY.

1. As a selective dye for the isolation of bacilli of the paratyphoid-enteritidis type, brilliant green exhibits a greater degree of specificity than malachite green (Höchst 120).

2. Brilliant green glucose broth, prepared according to methods described in this paper, constitutes an enrichment medium of marked selective propensity for the paratyphoid-enteritidis group. A greater degree of success was attained in isolating *B. paratyphosus* B and *B. enteritidis* from the feces of dogs fed with these bacilli by the use of this enrichment medium than with Loeffler's malachite green-nutrose-lactose agar medium. Feces containing so few paratyphoid or enteritidis bacilli that none of their colonies appeared on the Endo plates or even on the Loeffler plate medium gave pure or nearly pure cultures with this enrichment medium.

THE TOXICITY OF HUMAN TONSILS.*

GEORGE F. DICK AND W. H. BURMEISTER.

(From the Memorial Institute for Infectious Diseases, Chicago.)

The relation of the tonsils to diseases of other parts of the body is a much-discussed subject. Besides those affections in which the tonsils serve clearly as an atrium of infection, there are others in which the relationship is not so well understood. Among these, Kyle¹ emphasizes asthma. He considers this in the nature of a reflex neurosis but also considers the possibility of the absorption of toxic substances from the tonsils. Tschiasny² reports a case of recurrent bronchial asthma in which removal of the tonsils resulted in a cessation of the attacks. The frequency of convulsions in children and of so-called epileptiform attacks in young individuals with hyperplastic lymphadenoid systems has led to some speculation as to the relationship of this condition to such attacks. Here too the reflex and the toxic possibilities are those considered. Fignero³ reports a case of epilepsy in a boy of 13 who had no further attacks during the period of four years following the removal of adenoids. The idea of absorption of toxic substances through the tonsils is supported by the changes of chronic tonsillitis. Davis⁴ mentions the action of toxins as a possible explanation of the plasma cell infiltrations in tonsils.

The source of the supposed intoxication generally is said to be that of the bacteria associated with the tonsils. It is known, however, that the bacteria usually found in the tonsils do not form soluble toxins. Dold and Aoki⁵ have succeeded in forming anaphylatoxin from streptococci which are the commonest organisms found in chronic tonsillitis, but only with some difficulty, while others have failed entirely. It is evident that while the assumption of absorp-

* Received for publication July 1, 1913.

¹ *Diseases of the Nose and Throat*, Philadelphia, 1909.

² *Monatschr. f. Ohrenheilk. u. Laryngo-Rhinolog.*, 1913, 47, p. 356.

³ *Arch. de med. des enfants*, 1912, 15, p. 691.

⁴ *Jour. Infect. Dis.*, 1912, 10, p. 147.

⁵ *Ztschr. f. Immunitätsf.*, 1912, 13, p. 200.

tion of toxic substances from the tonsil is common, the problem of the existence and nature of the toxic substances is still unsolved. It would seem therefore that this subject is worthy of further investigation.

The work, the results of which we now report, was undertaken to determine first, whether or not there are toxic substances in the tonsils; second, if present, the nature of those substances, and third, the factors influencing the degree of toxicity.

The material was obtained from a series of 32 tonsillectomies.¹ The tonsils were received in sterile gauze. Extracts were then made by grinding them in a mortar with 10 c.c. of salt solution. The extract thus obtained was either centrifuged or filtered through paper and examined bacteriologically as follows: blood agar plates were made by adding one cubic centimeter of goat blood to seven to nine cubic centimeters of agar; the plates were allowed to harden, and one drop of extract to be examined was smeared over the surface of two plates by means of a platinum spatula; after incubating, 18-24 colonies were examined grossly and microscopically.

The following will serve as examples of these experiments:

A rabbit received two cubic centimeters of tonsillar extract intravenously (marginal veins of ear). After one and one-half minutes it urinated, jumped several times, fell on one side, jumped again, had clonic movements (opisthotonos), and died. Quivering contractions of muscles could be felt even after the animal lay on its side apparently dead. On examination 20 minutes later both ventricles were in contraction; the right muscle in rhythmic contractions, lungs not distended; no pulmonary hemorrhage, heart's blood fluid.

A guinea-pig received four cubic centimeters of extract intravenously. Temperature before, 102° F. Sneezing followed by convulsions within 30 seconds after injection, temperature after one and one-half minutes, 100.7° F.; respiration ceased after two and one-half minutes. Postmortem, typical pulmonary distensions with subpleural hemorrhages; heart contracting feebly but in rhythm, right ventricle slightly dilated; heart's blood fluid.

Female dog, weight 9 kilograms, received five cubic centimeters intravenously. Convulsions, urinated, defecated, vomited within two minutes, recovery within five minutes.

It was noted that of animals injected with toxic extracts the rabbit usually exhibited, besides the symptoms noted, a marked exophthalmos and contracted pupils. Postmortem examinations

¹We wish to thank Dr. A. A. Hayden, of St. Joseph's Hospital, Chicago, for his courtesy in providing us with material.

were made in nearly all cases and the changes are all illustrated by the protocols given. In no instances were evidences of embolism or thrombosis found. The coagulation of the blood was delayed.

The extracts were prepared and injected in some cases immediately upon removal of the tonsils. A few of them were prepared after freezing the tonsils for 24 hours; most of them were made a few hours after removal. The toxicity was apparently not affected by these varying conditions.

It is evident from the protocols that the symptoms and changes in the animals resemble very much the conditions found in anaphylactic shock in rabbits,¹ guinea-pigs,² and dogs.³

Friedberger⁴ found that anaphylatoxin was destroyed by heating to 65° C. Heating our extracts to 60°-65° C. diminished their toxicity markedly without, however, rendering them entirely harmless since a rabbit, which received two cubic centimeters intravenously, had convulsions within 30 seconds, and died within two minutes. The same quantity of the same extract, heated to 65° C., for 30 minutes, produced uneasiness after one minute, slight twitching after three minutes, mild convulsive movements after five minutes, and death.

The effect of passing of tonsillar extracts through Berkefeld filters was tried as follows: a rabbit, which received one cubic centimeter intravenously, had convulsions and died within one minute; a guinea-pig received one cubic centimeter intracardially and showed jerky respirations; a guinea-pig received one cubic centimeter of the Berkefeld filtrate intravenously and showed no symptoms.

Not infrequently, as is also illustrated by this result, it was found that some extracts which were fatal for rabbits were far less toxic for guinea-pigs.

Friedberger and Hartoch⁵ have also shown that hypertonic salt solution previously injected protects animals against anaphylactic shock. Hypertonic salt solution also protects animals against the action of tonsil extracts. While two cubic centimeters of a

¹ Auer, *Jour. Exper. Med.*, 1911, 14, p. 476.

² Auer and Lewis, *Jour. Exper. Med.*, 1910, 12, p. 151.

³ Schittenhelm and Weichardt, *München. med. Wchnschr.*, 1910, 58, p. 841.

⁴ *Ztschr. f. Immunitätsf.*, 1910, 7, p. 751.

⁵ *Ibid.*, 1909, 3, p. 587.

certain extract killed immediately, the injection of one and one-half cubic centimeters of 25 per cent NaCl solution intravenously caused a few clonic convulsions within one minute or so, followed by complete recovery, and protected fully against two cubic centimeters of tonsil extract given immediately after the convulsion.

Friedberger and Mita¹ found that by injecting slowly, twice the fatal dose of anaphylatoxin can be given. This is true of toxic extracts of tonsil also: a rabbit was injected at intervals of a few minutes with 0.5 c.c. doses. In all, six doses were given, a total of three cubic centimeters. The animal remained unaffected. The same extract injected immediately afterward into two animals in doses of one and one-half and two cubic centimeters proved rapidly fatal to both.

The protective inoculation of atropin in anaphylactic shock has been noted by various investigators, notably Auer,² and the effect of atropin with respect to toxic tonsillar extracts was tried as follows: a large rabbit was injected with three cubic centimeters of extract. Convulsions and death followed within four minutes. A much smaller rabbit was injected with three cubic centimeters four minutes after an intravenous injection of 0.02 of a grain of atropin. At the time of injection of extract the pupils were dilated. Within one minute the pupils contracted. The animal died without convulsions. The atropin, then, seemed to exert an antispasmodic effect but did not prevent death.

It seems clear that the extracts of tonsils contain a substance which manifests many of the characteristics of the so-called anaphylatoxin of Friedberger. In this connection the toxic extracts of normal organs investigated particularly by Dold³ may be mentioned. He found that such extracts produced harm by causing coagulation of the blood and were easily affected by heat, thus differing from the extracts of tonsils.

A few tonsils were apparently harmless in the doses of extract used. A search for the underlying factors was made by comparing the toxicity with the bacterial flora, structural changes, and the amount of sediment, i.e., parenchyma, obtainable. The bacterial

¹ *Deutsch. med. Wchnschr.*, 1912, 38, p. 204.

² *Ztschr. f. Immunitätsf.*, 1912, 12, p. 235.

³ *Ztschr. f. Immunitätsf.*, 1911, 10, p. 53.

TABLE 1.

Case	Toxicity c.c.	Predominating Bacteria	Bacteria Second in Number	Bacteria Third in Number	Bacteria Fourth in Number
1...	1 D	M. catarrhalis	Pneumococcus	Sta. albus
2...	1 D	Hemolytic streptococci, large colonies, short chains
3...	1 2 1.5 6 D	Hemolytic streptococci, large colonies	Hemolytic streptococci, punctate colonies very few
4...	0.5 D	Str. viridans	Sta. albus	Many punctate hemolytic streptococci
5...	2.5 D	Hemolytic streptococci, large colonies	Str. viridans	Sta. albus
6...	1.5 D	Hemolytic streptococcus, large colonies	Hemolytic streptococcus, punctate colonies
7...	3 D	Str. viridans	Many hemolytic streptococci, punctate colonies	M. catarrhalis	Pneumococcus
8...	2 5 7 D	Pneumococcus	M. catarrhalis	Str. viridans
9...	6 L	Pneumococcus	Str. viridans
10...	7 L	Str. viridans	Pneumococcus
11...	6 L	Hemolytic streptococcus, flat wrinkled colonies
12...	2 D	Str. viridans	Sta. albus	Hemolytic streptococcus
13...	2.5 D	Hemolytic streptococcus	Str. viridans	Sta. albus
14...	1 D	Hemolytic streptococcus	Str. viridans
15...	6 L	Str. viridans	Friedländer's bacillus
16...	1 L C	Hemolytic streptococcus	Sta. albus
17...	1.5 D	Pneumococcus	Hemolytic streptococcus	M. catarrhalis
18...	2 D	Pleomorphic gram-organism	Staphylococcus, small colonies
19...	6 L	Str. viridans	Pneumococcus	Sta. albus	Gram and diplococcus, no effect on blood
20...	2 D	Hemolytic staphylococcus	Pneumococcus
21...	7 L	Pneumococcus	Pseudodiphtheria bacillus	Str. viridans	Friedländer's bacillus
22...	2.5 L C	Hemolytic streptococcus	Pneumococcus
23...	1 D	Pneumococcus	Hemolytic staphylococcus

TABLE 1.—*Continued.*

Case	Toxicity c.c.	Predominating Bacteria	Bacteria Second in Number	Bacteria Third in Number	Bacteria Fourth in Number
24...	1 D	Hemolytic streptococcus, small colonies	Pneumococcus
25...	2 D	Str. viridans	Pneumococcus	M. tetragena	Friedländer's bacillus
26...	2 D	Hemolytic staphylococcus	Streptococcus, no effect on blood
27...	1 D	M. catarrhalis	Hemolytic strep- tococci, small colonies
28...	2 D	Pleomorphic gram-diplococcus	Hemolytic strep- toccus	Pneumococcus
29...	7 L	Hemolytic streptococci, large colonies wrinkled	Large diplococcus	Str. viridans
30...	7 L	Streptococcus, large, flat, wrinkled colonies	Pneumococcus	Streptococcus, small hemolytic colonies
31...	5 C dog	Hemolytic streptococcus, small punctate colonies	M. tetragena
32...	2 D	Plates	Contaminated
33...	5 L	Streptococci, no effect on blood	M. catarrhalis	Str. viridans

D indicates death following injection; L indicates that the animal lived; C, convulsions.

flora was the only factor that seemed to bear any relationship to the toxicity of the extract. Space will therefore be given to a consideration of the bacteriology only. An idea of the connection between bacteria and toxicity can best be obtained from Table 1.

In the 10 cases in which relatively non-toxic extracts were obtained there was only one in which typical punctate hemolytic streptococcus colonies were present. In the remaining 23, from which highly toxic extracts were obtained, punctate, hemolytic, streptococcus colonies occurred 15 times in large numbers. In three additional cases large colonies of hemolytic streptococci were found. In the remaining five a hemolytic staphylococcus was found three times. Hence the toxic extracts were usually obtained when typical hemolytic streptococci were found on the plates in large or predominating numbers.

As previously stated, investigation has shown that anaphylatoxin is obtained with difficulty from streptococci; indeed Aronson¹

¹ *Berl. klin. Wchnschr.*, 1912, 49, p. 204.

maintains that they never yield an acutely fatal toxin. The prevalence of these organisms in our most toxic extracts led us to an attempt to obtain anaphylatoxin from the organisms grown on the plates by the method of Friedberger. In only one out of six attempts was a toxic substance obtained. It would therefore seem that if these substances are formed from the bacteria present, then the tonsils must present conditions very favorable to their formation.

CONCLUSIONS.

1. Extracts of tonsils are acutely toxic for animals.
2. These substances affect animals in a manner similar to that of anaphylatoxin.
3. The relation of the toxicity of tonsil extracts to the bacterial flora demands further study. The results of this work, however, would indicate that as a rule extracts of those tonsils are most toxic which are associated with hemolytic streptococci.

A SPIROCHETE ASSOCIATED WITH INFECTIONS OF THE ACCESSORY SINUSES.*

RUTH TUNNICLIFF.

(From the Memorial Institute for Infectious Diseases, Chicago.)

In examining the smears from the nose of 32 cases of acute and chronic rhinitis, a spirochete, staining deeply with carbol-gentian-violet was observed in seven cases. In six of these it was found in connection with infections of the accessory sinuses. In the other case no history could be obtained except that of a severe acute rhinitis. In an infection of the frontal sinuses this spirochete appeared to be the only organism present in the smears.



FIG. 1.—Smear from nose. Antrum infection.
× 1200. Carbol-gentian-violet.

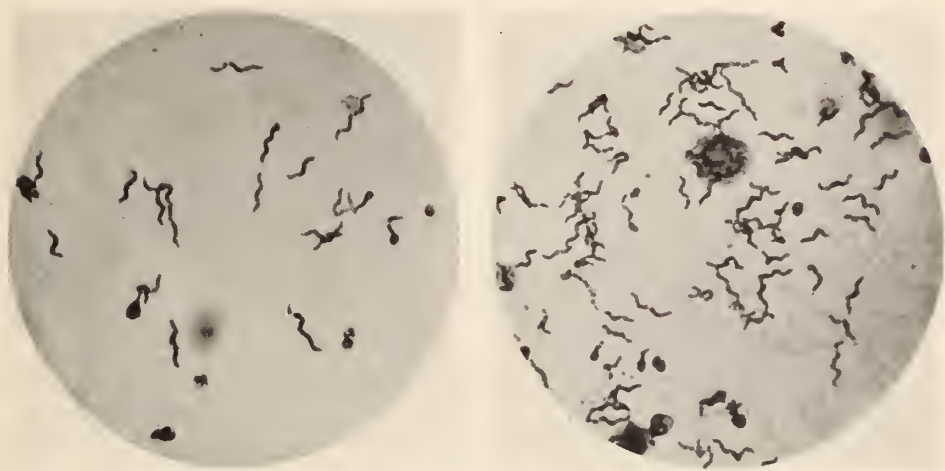
The organism has two or three regular curves, the ends being as a rule pointed and generally parallel to the long axis (Fig. 1). Inside and protruding from them are occasionally seen deeply staining bodies about three times the width of the spirochete, which measures from two and one-half to five microns in length

and one-half of one micron in width. It is a strict anaerobe and was isolated in pure culture on goat blood agar in large test tubes, the agar being slightly alkaline to phenolphthalein. The cultures were grown anaerobically according to the method of Wright, by saturating the cotton stopper with a strong solution of pyrogalllic acid in a five per cent solution of sodium hydroxid, closing the tube with a tightly fitting cork and sealing it with paraffin. The growth appeared on the surface after seven days' incubation as a dull,

* Received for publication June 12, 1913.

radiating growth, later also in the fluid of condensation. In subcultures it formed raised, pin point colonies near the fluid of condensation, the rest of the growth being dull and rather profuse. The growth at first was mucoid, but lost this characteristic after being transplanted several times. The organism also grew in ascites and dextrose agar but produced no change in the media. There was no odor from the cultures.

In cultures (Figs. 2 and 3) the spirochetes measure about six microns in length and seven-tenths of a micron in width. They show from two to four curves, sometimes more, and have a tendency



FIGS. 2 and 3.—Pure cultures, eight days old. $\times 1200$. Carbol-gentian-violet.

to be irregular in shape but often appear just like those in the mucus. Chains of two or three spirochetes joined by a fine filament are occasionally seen. They frequently stain irregularly. Oval, round, only slightly curved bacillary forms are seen. Deeply staining bodies and rings may be attached to the ends, and with the Giemsa stain two or three red stained bodies are brought out inside the spirochetes, which stain fairly well with methylene blue, carbolthionin, carbol-fuchsin, and retain Gram's stain. The spirochete possesses many curved flagella on its ends and sides; the free round bodies also appear to possess flagella. After growing about seven days the spirochete disintegrates suddenly, leaving many poorly

stained bodies as a result. The organism is commonly motile having a swift darting movement and some rotary motion; the refractile bodies present are also actively motile.

It is not pathogenic when injected into the nose of a dog.

This spirochete does not look like the "irregularis" and "undulata" mentioned by Gerber,¹ as having been seen in a case of rhinitis sicca and in an ulcer of the nose, nor does it resemble the fine spirochetes sometimes seen in the nose, which look like those in the mouth. Culturally this spirochete does not appear to correspond to any of the spirochetes already isolated in pure culture. The two spirochetes which this organism resembles culturally are *Treponema macrodentium* and *Spirochaeta refringens*, both isolated in pure culture by Noguchi.² The first differs from this nose spirochete in the greater length of its viability, the swinging or vigorously vibrating movement, its greater length in old cultures, and the fact that it requires tissue for its growth, produces a hazy growth and possesses flagella-like projections only on one or both ends. *Spirochaeta refringens* differs from it in its greater length; its shape, the middle being as a rule merely wavy, the two extremities being more regularly and deeply curved; in forming hazy colonies; in possessing fine, long, more or less elastic projections, which have minute curves; and in the motion of the flagella-like projections rotating around the spirochete, the spirochete remaining motionless, or traversed by waves.

The significance of this nose spirochete is yet to be determined.

¹ *Centralbl. f. Bakteriöl.*, 1910, 56, p. 508.

² *Jour. Exper. Med.*, 1912, 15, pp. 81 and 466.

AN ANAEROBIC ORGANISM ASSOCIATED WITH ACUTE RHINITIS.*

RUTH TUNNICLIFF.

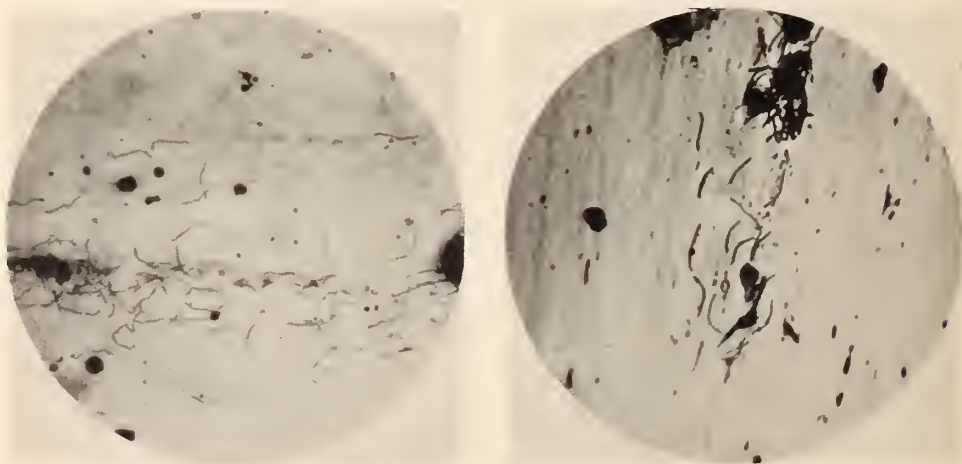
(From the Memorial Institute for Infectious Diseases, Chicago.)

Many efforts have been made to determine the cause of acute rhinitis and a large variety of organisms have been observed, but so far as I have been able to determine all the investigations have been made with aerobic methods.

The organism now described has been found during the early stages of acute coryza, while the discharge is mucoid in character. At this time few, often no other, bacteria are present in the smears, while this organism is often found in large numbers. As the mucus disappears or the discharge becomes purulent, it decreases in number and finally disappears while other bacteria increase in number. It has been observed in all cases of acute rhinitis studied, 25 different persons having been examined during the acute attack, one during each of four attacks and another during each of five, so that altogether I have made observations during 32 distinct attacks. Many examinations were made during the course of the infection in nine of these cases. In the case of an accompanying pharyngitis, tonsillitis or bronchitis, the organism is also found, sometimes in large numbers in the sputum and mucus of the throat. It was also seen in a case of acute pharyngitis unaccompanied by rhinitis. It was present in small numbers in the nose in five cases examined when the rhinitis was disappearing. It was not found in 20 normal persons, in one case of suspected syphilis and one case of diphtheria. A few were observed in one normal nose. The same organism has been seen in one case of chronic pharyngitis, being practically the only organism present, and in three cases of chronic rhinitis. In some of the lower animals it appears to be present since it was found in the noses of several normal guinea-pigs and rabbits examined, but seen only once in normal dogs. It is not present in the purulent discharge common in rabbits. This same organism was observed in the mucoid discharge from the nose of a dog.

* Received for publication June 22, 1913.

In order to find the organisms a fair amount of mucus should be spread on a slide and often a large number of fields must be examined. They generally appear in clumps, but many are found separate. Carbol-gentian-violet and carbol-fuchsin are the best stains for this organism; but it generally stains rather faintly even with these and it does not stain by Gram's method, methylene blue, carbol-thronin or the Giemsa stain. It may be seen by dark field illumination. Some of the organisms seem to be flexible and move a little, but most of them appear immobile, which may be due to the large amount of mucus present. They vary from five to eight



FIGS. 1 and 2.—Smears from nose. Acute rhinitis. $\times 1000$. Carbol-gentian violet.

microns in length and from one-third to one-half of a micron in width; the ends are pointed or slightly rounded; generally the bodies are slightly curved, but may be straight, wavy, or bent over at one end. A ring and an enlargement in the form of a ball are occasionally seen at one extremity. Sometimes an arrangement in rosettes and bunches occurs, the organisms radiating from a central mass.

This organism is a strict anaerobe growing at 37° C. slowly—at least at first. I have isolated it in pure culture eight times, four times from the human nose, three times from the throat and once from the nose of a normal rabbit. Dr. George F. Dick has isolated

it once from a case of chronic rhinitis. It has been cultivated several other times but not isolated in pure culture. It was isolated on the surface of goat blood agar in large test tubes, the agar being slightly alkaline to phenolphthalein. The cultures were made anaerobic by the Wright method of saturating the cotton stopper with a strong solution of pyrogallol acid and a five per cent sodium hydroxide solution, closing the tube with a tightly fitting cork and sealing it with paraffin. The colonies appear in from five days to a month, usually in about seven days, as small, round, dull growths. After several transfers growth becomes more active and less dull in appearance and in old cultures it becomes thick and greenish yellow, or brown in color. Growth also occurs in dextrose agar, dextrose and horse serum agar, ascites agar, and ascites agar with rabbit kidney tissue, along the stab canal as a distinct creamy growth, but produces no haziness. On plain agar there is but little growth, sometimes none, and there is no growth in plain broth or in milk and generally none on the surface of Loeffler's blood serum. When it grows in dextrose broth, the growth is flocculent. The various strains agree culturally except in their growth on potato, two producing a yellow growth, one a cream colored growth in the fluid only, the one from the rabbit, white colonies, and the others no growth at all. Evidently the age of the culture and the number of organisms inoculated are important in determining growth, the same strains varying, sometimes growing in a given medium, sometimes not.

Examined by dark field illumination the organism from cultures is flexible and motile, but loses motion quickly. It is progressive, tumbling, and vibratory. Attached to the ends and sides and also free are light refractile bodies, the free ones being actively motile.

In cultures the organisms measure from three to eight microns in length and one-third to one-half of a micron in width. The organism now stains a little more deeply than in the smears from the mucus, but remains gram-negative. It varies in its ability to stain with methylene blue, some cultures not staining when first isolated though staining later, other strains staining when first isolated. Organisms grown in dextrose agar and in the fluid of condensation of Loeffler's blood serum show deeply staining bands.

Morphologically the organisms from cultures look like the organisms found in the smears from the mucus, but after long cultivation there is a tendency in some strains to become larger, straighter and less bunched, while in early culture they are delicate, straight, slightly curved, some very wavy and some showing a radiating arrangement. Darkly staining bodies are seen attached to the ends and sides and also free; and an organism with a ring at one end is occasionally observed. Forms which are especially curved are seen in old cultures. Flagella have not yet been demonstrated. The organism looks a good deal like a spirochete, but

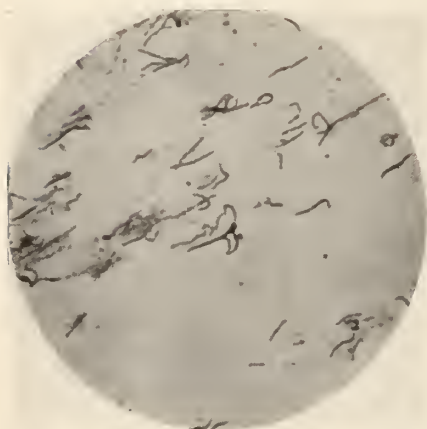


FIG. 3.—Pure culture, one month old. $\times 1200$. Carbol-gentian-violet.

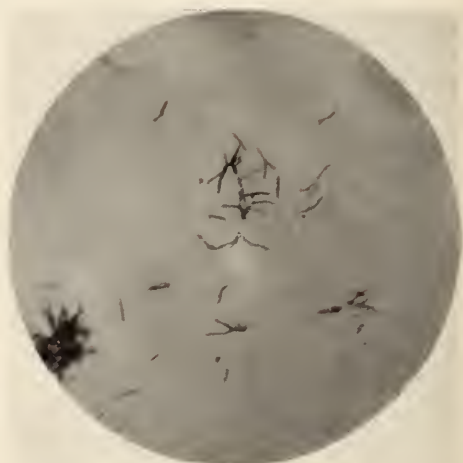


FIG. 4.—Pure culture, five days old. $\times 1000$. Carbol-gentian-violet.

culturally it seems more like a bacillus and whether it is a spirochete or a bacillus must be decided later.

These organisms in the mucus may be distinguished from cilia, by staining with a watery solution of eosin, the organisms not staining, and by the growth of the organism in cultures. They differ from fusiform bacilli, in not staining so distinctly, in being more delicate and not fusiform in shape, and in their motility. Culturally after they have been grown through several generations and have lost their dull appearance, their growth is much like that of fusiform bacilli. Morphologically and in their staining reactions they resemble the "nadel-förmigen bakterien" of Shmamine,¹ asso-

¹ *Centralbl. f. Bakteriöl., I Orig.*, 1912, 65, p. 311.

ciated with *Spirochaeta pallida*, but they differ from them in not forming the spirochete looking forms until grown several weeks, in being motile and in their growth, the "nadel-förmigen bakterien" producing a growth identical with that of the *Spirochaeta pallida*.

During the acute stage of rhinitis the opsonic index to this organism is low, rising as the infection subsides.

A slight rhinitis has been produced several times in human subjects (twice in myself, once in a laboratory assistant) by swabbing a nose which was free from this organism, with a pure culture. A pharyngitis accompanied the rhinitis twice. The infection began from six to eight hours after the inoculation and lasted about 48 hours. The organisms were present in fairly large numbers in the nose and pharynx (in the cases with pharyngitis) 18 hours after the inoculation and persisted for three days in two of the cases. Cultures were made twice and the organisms isolated in pure culture both times. The opsonic index was taken during two of these infections. Both times it fell below normal, rising high above normal as the infection disappeared.

A slight rhinitis has been produced in a dog also, the organisms disappearing from the nose in 24 hours. The organisms were grown through several generations, but not isolated in pure culture.

Two patients have been injected with the killed organisms to determine the number of organisms and the frequency of injections necessary to produce a definite reaction and not with any idea of drawing conclusions as to the value of the vaccine. The vaccine was made in the usual way, heating the suspension at 60° C. for one hour. The organism had been isolated from a patient with acute rhinitis. This patient had recurring attacks of acute coryza and pharyngitis, the organisms being found in the nose and pharynx only during the infections. Three injections were given, one week elapsing between each; 50,000 only were injected the first time, 100,000 the second and 200,000 the third. After each injection the negative phase of the opsonic index has been less and the rise higher, being 1.5, 2.5 and 3.5 respectively. After each injection there was a slight rhinitis and pharyngitis, but no severe infections have occurred.

The other patient had a severe persistent rhinitis with a profuse mucoid discharge and severe attacks of sneezing. He had had an

operation on the nose and had used various sprays without receiving any relief. These organisms were present in enormous numbers in the discharge from the nose and were the predominating organisms. Before the injections his opsonic index was 0.6. He received at the first injection 50,000 killed organisms and the number was increased each time, about four days elapsing between each injection. After the first, the index rose to 2.1 within 24 hours and remained high for four days, the discharge and sneezing almost disappearing. The second injection (200,000) was given when the index had fallen to 0.4 and after this injection the index did not rise above normal and the patient felt less well and had more discharge. After the third injection (165,000), four days after the second, he was much better in every way, having practically no discharge and no sneezing attacks, the index reaching 1.9. Five days after the fourth injection (250,000) he had several severe attacks of sneezing and a large amount of discharge which stopped after the fifth injection (350,000), the patient's general condition also being much improved. It is too soon to know whether the improvement will be permanent and how long the treatment must be continued, but the small doses were evidently sufficient to produce definite changes in the blood and symptoms.

Whether this anaerobe is the cause of acute rhinitis must be determined by further investigation. My observations show that in the cases of acute rhinitis examined, it was present constantly during the early stage of the coryza and disappeared as the infection subsided.

AN ANAEROBIC BACILLUS ISOLATED FROM A CASE OF CHRONIC BRONCHITIS.*

RUTH TUNNICLIFF.

(*From the Memorial Institute for Infectious Diseases, Chicago.*)

In making bacteriological examinations of the sputum from a case of chronic bronchitis, suspected of being tuberculous, an anaerobic bacillus was found to be the predominating organism. So far as I have been able to determine, this bacillus has not been previously described.

The patient is a man 62 years old. Until four years of age he was perfectly healthy. At that time he had a severe attack of pneumonia, followed by repeated attacks of lobar and bronchopneumonia.

About 40 years ago the patient began to suffer probably from tuberculosis, having repeated attacks of chills, fever, and sweating for eight years, getting better for a year and then worse again. At this time he had a very severe attack of lobar pneumonia, and a pronounced pleurisy with effusion. Since then he has suffered from similar attacks of pleurisy with more or less bronchitis; 19 years ago he had a very severe attack of pleurisy with effusions lasting several weeks; these repeated attacks of bronchitis and pleurisy, which always disappeared after a few weeks, persisted until four years ago, since which time only bronchitis has been troublesome. Six months ago the patient was ill with an attack of bronchitis, much worse than those previous, and continued with a profuse expectoration until the vaccine treatment now to be described was employed.

The bacillus in the sputum is about two microns long by one-fourth micron in width. It was found in enormous numbers generally in clumps. Some bacilli were seen inside of leukocytes. They were found as a rule in greyish masses in the sputum. Altho resembling tubercle bacilli a good deal, they were not acidfast nor did they retain Gram's stain. They stained uniformly

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and deeply with carbol-gentian-violet, faintly with methylene blue, having a beaded appearance, as they did with carbol-fuchsin. With Giemsa they stained blue.

The bacillus is a strict anaerobe, growing slowly, especially during the first generations. The first time it was isolated, growth did not begin until the fifteenth day after inoculation. It was isolated on goat blood agar, the agar being slightly alkaline to phenolphthalein. It grew in the fluid of condensation as a thick mucoid mass. On the surface of the agar profuse thick yellowish colonies appeared, containing the bacillus mixed with other bacteria. In subcultures the bacillus was isolated in pure culture, the colonies now appearing after five days' incubation. The colonies are round, convex and white, looking like pearls. As they grow older they become yellower in color. They are hard and can be removed only by also removing the agar. They may be pierced by a stiff needle. They grow in the fluid of condensation as a greyish-white mucoid mass, or the organisms may grow as distinct balls, held in a mucoid mass. In subcultures the colonies may appear in 48 hours as pinpoint, hard, dull colonies, increasing gradually in size and becoming white as they enlarge. The bacillus was isolated in pure culture a second time, the colonies appearing then on the eighth day, as separate pearl-white hard colonies. The bacillus after five months' isolation is still a strict anaerobe and has not become acidfast.

The bacillus grows in 2 per cent dextrose broth in a creamy irregular mucoid mass, the fluid remaining perfectly clear. At first it would not grow on the surface of ascites agar and Loeffler's blood serum, only growing in the fluid of condensation as in the dextrose broth. The colonies on ascites and glycerin agar appear like those on blood agar, but those on Loeffler's blood serum are a little softer. On potato the colonies are a little more irregular in shape, especially as they get older. The edge of the colonies as a rule is smooth. The colonies adhere to the media, but do not appear to grow far into it. The bacillus does not grow on plain agar, plain broth, milk, dextrose or mannite agar.

The bacillus is not motile and does not possess a capsule. When grown on potato and Loeffler's blood serum, it appears larger than when grown on blood agar and is more curved and shows

many bizarre forms. When grown on alkaline goat-blood agar or ordinary blood agar, it appears as a short, fairly thick bacillus of the same size and staining properties as the bacillus found in the smears from the sputum. Filaments are sometimes seen in the cultures. They are also seen in the sputum.

If inoculated in large numbers the bacillus appears to be toxic to guinea-pigs, but not pathogenic. The sputum injected into a guinea-pig did not produce any lesions. A guinea-pig was injected in the right lung with the growth in the fluid of condensation from

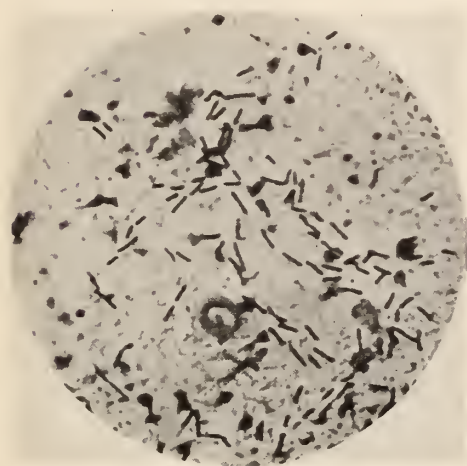


FIG. 1.—Smear from sputum. $\times 1200$. Carbol-gentian-violet.



FIG. 2.—Pure culture, three days old. $\times 1200$. Carbol-gentian-violet.

two tubes of Loeffler's blood serum grown 48 hours. Eighteen days later the same pig was injected in the left lung with the growth in the fluid of condensation of three tubes of Loeffler's blood serum. Immediately after this injection the guinea-pig had great difficulty in breathing, lying on the right side and twitching considerably. It had some trouble in breathing and appeared sick for four days. Ten days after the second injection the guinea-pig was killed. Areas of atelectasis were found in both sides, and a small area of congestion was seen in the middle right lobe. This proved to be chronic bronchopneumonia.

Another guinea-pig (small) was injected in the lung with the

growth in the fluid of condensation of two tubes of Loeffler's blood serum. In seven days it died in convulsions. No pathological lesions could be found.

Repeated intraperitoneal injections in a guinea-pig have so far produced no effect.

On account of the chronic condition of the bronchitis, the presence of cavities in the lung as determined by physical examination, and the profuse expectoration, it was thought a vaccine of this bacillus might prove of value. The colonies were dug out of the blood agar, suspended in normal salt solution, and ground in a sterile mortar. This suspension was centrifuged to remove the pieces of agar and then heated for one hour at 60° C. Its sterility was determined by cultures and animal inoculation. The patient's opsonic index was determined before and after the inoculations. The bacilli for these experiments were also ground in a mortar to get suitable suspensions. The bacilli are practically not taken up at all in the presence of normal serum, as a rule about four cells out of 50 taking part in phagocytosis. The patient had an index slightly higher than normal before the injections began. After each injection the changes in the serum were most marked, as seen both in the number of bacteria ingested by each leukocyte, and by the number of cells taking part in phagocytosis. The indices after the injections ranged from 18 to 38. After the first two injections there was a negative phase lasting four days after the first, and one day after the second. No observations were made after the third injection. There was no negative phase as indicated by the opsonic index, after the fourth injection.

Five hundred thousand killed bacilli were injected intramuscularly each time, about one month elapsing between each injection. The symptoms were marked after each injection and hence a larger dose was not considered advisable. After each injection there was headache, malaise, sometimes chilliness, the appearance of rales (sibilant and mucous) with increased cough and expectoration. After the first and third injections a little blood appeared in the sputum. About four days after the first injection the patient began to feel much better, sleeping better than for weeks, and having much less bronchitis and therefore decreased expectoration.

After the second injection when the index rose to 38, the patient was better than he had been for six months. The amount of sputum was greatly decreased, being less than one-tenth of the amount it had been before the injections. The sputum was more mucoid and less purulent in character. After the third injection the patient had about the same reaction as after the first. When this had passed off the bronchitis was much improved and he felt better again and gained in weight.

The bacilli are still present in the sputum, altho the amount of sputum is greatly reduced.

Whether the vaccine will be able to overcome the infection in the cavities is, of course, doubtful. There can be little doubt, however, that the injections have greatly improved the condition of the patient, the bronchitis being much diminished.

The large number of bacilli present in the sputum, the decided local reaction in the lungs after each injection, and the improvement in the bronchitis after the vaccine treatment would indicate that this organism may have some causal relation with the bronchial infection.

STUDIES ON THE CULTIVATION OF THE VIRUS OF VACCINIA.*†

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Many investigators have shown that the virus of vaccinia, except in a dried form, retains its virulence but a short time at 37° C. The virus is usually found inactive after 9 to 13 days' incubation, altho under special conditions it has been kept as long as 16 to 21 days. No definite subcultures, however, have been obtainable.

We wish to refer in some detail, in this connection, to the work of Williams and Flournoy.¹ They inoculated with vaccine virus the corneas of rabbits, which were removed 24 hours after inoculation and placed in tubes of ascitic broth. The tubes were incubated at 36° C. After 24 hours' incubation the loosened epithelial cells formed an emulsion in the fluid. The inoculation of this emulsion on the skin of a rabbit gave a typical eruption. In one experiment, in which transfers to fresh broth were made on alternate days, the inoculations of the second and third transfers on the cornea of a rabbit produced typical ulcers. Inoculations from the fifth and sixth transfers on the skin of a calf gave a few scattered pustules; inoculations of subsequent transfers were negative. The inoculations of the fourth, fifth, and sixth transfers on the skin of rabbits were negative. The authors attribute the activity of the transfers to a carrying over of the original virus, since they saw no evidence of multiplication. The total period of incubation of the virus in their experiments was from 10 to 12 days.

EXPERIMENTAL WORK.

In previous work with the virus of rabies, done in conjunction with D. W. Poor,² we applied Harrison's method of growing

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¹ *Studies from the Rockefeller Institute*, 1905, 3, p. 146.

² *Jour. Infect. Dis.*, 1912, 11, p. 459.

tissue *in vitro* to brain tissue and were able to produce inclusions in normal ganglion cells closely resembling certain small forms of Negri bodies, but were not able to cause any multiplication of the virus of rabies. After these results with rabies, we decided to continue the work with Harrison's method, applying it in the present experiments to the virus of vaccinia incubated with corneal tissue and plasma, again, with two objects in view: the possible production of vaccine bodies *in vitro*, and the possible cultivation of the virus of vaccinia outside of the body.

The virus was taken from the stock of glycerinated, carbolyzed, calf virus as distributed by the Board of Health of New York City. It was dialyzed through collodion sacs by the method of Poor and Steinhardt.¹ It was usually allowed to stand in the icebox over night, thus permitting the coarse particles to settle. The supernatant fluid was drawn off and diluted with Ringer's or physiological salt solution. By this means a fairly uniform emulsion is obtained.

Technic.—Small pieces of rabbit or guinea-pig cornea were placed for a few minutes in this weak emulsion of virus. The pieces were then transferred with a small quantity of the virus to cover-glasses to which drops of rabbit or guinea-pig blood plasma were added. The cover-glasses were immediately inverted and sealed over hollow-ground slides, which were incubated at 37° C. To control the microscopic work, similar preparations were put up without virus, and to control the necessity of the cornea in the animal inoculation work, pieces of paraffin were substituted for cornea in a series of preparations.

Microscopic studies.—Approximately 100 hanging-drop preparations upon five series of experiments were studied histologically, using several methods of fixation and staining. Examinations were also made on fresh unstained preparations with light and dark field illumination. The preparations were stained after varying periods of incubation. As a rule, four slides were taken out at once and studied in several ways. To fix a specimen, the cover-glass with the attached clot was dropped directly into a dish of the fixing fluid unless the fixative was bichlorid, when, according to the method of

¹ *Jour. Infect. Dis.*, 1913, 12, p. 202.

Lambert,¹ it was first soaked for 1-2 hours in salt or Ringer's solution to prevent the precipitation of proteins giving subsequently a cloudy specimen. The subsequent staining was carried out as tho the specimen were a paraffin or celloidin section attached to a slide, except that on account of the thickness of the hanging drop a longer time is required in the process.

Briefly stated, the methods employed were: (1) Zenker fixation, followed by eosin-methylene blue stain; (2) formalin fixation, Sudan III iron hematoxylin stain; (3) Giemsa's stain with and without previous fixation; (4) eosin-methylene blue without previous fixation; (5) unstained preparations with direct and dark field illumination.

The results of these studies may be given in a few words. The corneal epithelium shows an active lateral spreading through the clot, forming sheets or groups of cells in the plasma. The cells early show an accumulation of fat in their cytoplasm, but may retain their form for several weeks even when not transferred to fresh plasma. Careful studies have failed to reveal any specific vaccine bodies in the preparations; only smaller, undifferentiated forms have been seen and these have been found in both the controls without virus and the virus preparations after incubation.

We have also studied the corneas of guinea-pigs, inoculated with virus, which were removed 24 hours later and put up in hanging drops as described above. Only the smaller forms of the vaccine bodies were found in the beginning, and no further developments were seen. These negative findings are of interest on account of the results of the animal inoculations of the incubated preparations as given below.

Altho we have observed numerous granules in the incubated preparations, these have not been sufficiently definite in character with the methods employed thus far in our studies to allow us as yet to make any positive statements in regard to them.

We wish here to record our indebtedness to Dr. A. W. Williams for her very helpful criticisms and suggestions in the microscopic work just described.

¹ *Bull. J. International Association of Medical Museums*, 1913.

ANIMAL INOCULATIONS.

To demonstrate the activity of the virus, we have adopted the method of Calmette and Guérin.¹ They have shown that the virus



FIG. 1.

FIG. 2.

FIG. 1.—Right side of rabbit inoculated with 9 unincubated cornea preparations. Result: 50-55 pustules.

FIG. 2.—Left side of rabbit inoculated with 9 cornea preparations, same series, incubated 14 days. Result: confluent raised eruption.

rubbed on the freshly shaven skin of the rabbit produces a typical vaccinia eruption, and, furthermore, by methods of dilution, that the

number of vesicles in the eruption indicates approximately the quantity of virus present. This method is generally used in commercial laboratories for standardizing the virus.

In our experiments, the skin of a rabbit was inoculated at once with a small number of *unincubated* preparations, and similar inoculations were made later with *incubated* preparations to determine whether or not there was an increase in the virus.

The "takes" with unincubated preparations, using eight different viruses, varied from 10-50 vesicles, whereas a similar number of preparations incubated 7-18 days gave in every instance an extensive confluent eruption, showing that a definite increase had taken place. Repetitions of these experiments with the different viruses have in all cases given the same results. As yet we have not found a virus that has not shown increased activity under these conditions of incubation. Preparations containing small pieces of paraffin showed, on the other hand, no increase in activity upon incubation.

The following protocols are examples of the results obtained.

Experiment R. 490.—A series of hanging drop plasma preparations were made with rabbit cornea and Virus A. A second series were made using small pieces of paraffin in place of cornea, care being taken to make the quantity of virus the same for both series.

A rabbit inoculated on the skin of the back with 12 unincubated preparations presented on the third to fifth day 9-10 vesicles. No later lesions appeared.

A rabbit inoculated on the left side with 9 cornea preparations incubated 7 days gave an extensive, raised, confluent take. We estimated that at least 200 pustules must have been present to have given a confluent eruption over the area involved (Fig. 4). The same rabbit was inoculated simultaneously on the right side with 9 paraffin preparations also incubated 7 days. Five pustules were observed on the fifth day, and there were no further developments. (See Fig. 3.)

Experiment G. 422.—Hanging-drop specimens were prepared with guinea-pig cornea and Virus B. Nine unincubated preparations used for immediate skin inoculation gave 20-30 lesions. (See Fig. 1.) An inoculation with a similar number of preparations incubated 14 days gave an extensive confluent take. (See Fig. 2.)

Experiment G.—Hanging-drop plasma preparations were made using guinea-pig cornea and Virus C and were incubated at 37° C. On the ninth day of incubation nine preparations were used for a rabbit skin vaccination. A typical rash appeared before the fifth day, in which 40-50 pustules were counted and on the seventh day, 55 pustules. Nine preparations incubated 18 days were used for vaccinating a second rabbit. An extensive, elevated, confluent rash resulted composed of at least 200-250 lesions. (See Fig. 2.)

Subcultures of the preparations have been made and successful skin inoculations have been obtained from the third transfer. The

virus has remained active after 34 days' incubation, and this obviously does not represent the limit of activity under such cultural



FIG. 3.

FIG. 4.

FIG. 3.—Right side of rabbit inoculated with 9 paraffin preparations incubated 7 days. Result: 5 pustules (other marks are crusts from shaving wounds).

FIG. 4.—Left side of rabbit inoculated with 9 cornea preparations incubated 7 days (same series as paraffin preparations). Result: confluent raised eruption. (Inoculation with 12 similar cornea preparations, same series, unincubated, was followed by a take of 10 pustules.)

conditions. In control experiments it was found that the virus alone was greatly weakened after a week's incubation, and that after

three weeks' incubation, inoculations on rabbits were negative altho much larger quantities of the virus were used than with the tissue preparations. Experiments undertaken with the object of obtaining a growth of the virus in test tubes have thus far given interesting and encouraging results.

Work on this point, with further experiments on subcultures, on the use of other organs and the necessity of living tissue in the culture experiments, methods of obtaining a sterile virus, immunity reactions *in vitro*, and the results of further microscopic studies will be described shortly in a later communication.

CONCLUSION.

From our work thus far on the application of Harrison's method to the cultivation of tissue *in vitro* to corneal tissue plus the virus of vaccinia, we are able to state that there is a multiplication of the virus of vaccinia altho no specific vaccine bodies are found in the preparations.

THE COMPLEMENT FIXATION TEST (GAY'S MODIFICATION OF THE BESREDKA METHOD) IN THE DIFFERENTIATION OF ACIDFAST BACILLI.*†

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(From the Laboratories of Pathology and Bacteriology, Tulane University of Louisiana, New Orleans, Louisiana.)

The great difficulty of substantiating any culture or culture-type obtained from the leprous nodule as the Hansen bacillus has necessitated the employment of the various tests and procedures in an endeavor to find a means to serve this purpose. Among these many efforts were included various serological methods. About 5,000 agglutination tests, utilizing the sera from 20 different cases of human leprosy and the sera of rabbits injected intravenously and subcutaneously with different acidfast bacilli, were performed. As the organisms used formed the more or less representative members of the acidfast group of both the saprophytic and pathogenic varieties, these studies apply to a large extent to this species. The cultures employed were the avian type of *B. tuberculosis*, the organisms considered as *B. lepra* by Kedrowski, Levy, Clegg, Karlinski, Duval, and the butter bacillus of Rabinowitsch, grass, dung, and the Smegma bacilli of Moeller; Korn I., *B. Phlei* and Grassburger's milk bacillus. The sera of these animals were also employed for the Bordet-Gengou reaction using most of the above enumerated organisms as antigens for homologous and cross-reactions. The results of these tests indicated that they were of little value, serving only as a part aid in differentiating certain groups but not affording reactions sufficiently clear-cut to serve for the identification of any culture as *B. lepra*. The fact that the serum tests were of no avail in the human cases when employing any of the various so-called lepra cultures did not, however, invalidate any of these strains as the Hansen bacillus, since bacillary suspensions prepared directly from the tissue nodules rich in bacilli

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† Read before the American Association of Pathologists and Bacteriologists, May 7, 1913, Washington, D.C.

yielded similar results. It was hoped that by the repeated injection of heavy suspensions into rabbits a serum of sufficiently high titre to serve for differentiation purposes might be produced and altho immune bodies of a fairly strong potency were stimulated for both the agglutination and complement binding tests, not only did these fail for definite specificity but the close analogy of the readings was striking. The results of the complement fixation test with these sera are shown in Table 1.

TABLE 1.

ANTIGENS	SERA					
	Non-chrome Duval	Chromogen Clegg	Chromogen Duval	Chromogen Currie	Chromogen Bayon	T. Hay
Non-chromogen Duval...	+++	-	+++	-	++	+
Chromogen Clegg.....	++	++	++	+	+	+
Chromogen Duval.....	++	++	++	+	+	+
Chromogen Currie.....	++	++	++	+	+	+
Chromogen Bayon.....	++	++	++	+	+++	+
Timothy Hay.....	++	+	+	+	+	+

Reagents used: Immune serum, 0.15 c.c.; complement, 0.5 c.c. of 10 per cent dilution of fresh guinea-pig serum; antigen, 0.5 c.c. of suspension of living bacilli (about 5,000,000,000 per c.c.); hemolytic system, 0.5 c.c. of 5 per cent suspension of sheep red blood cells and 2 M.H.D. of specific hemolysin.

Controls: Sera. No hemolytic nor anticomplementary property when using double quantities; antigens.

The inference from this work was that the antigenic substances used for immunization were so similar in composition that they stimulated antibodies too closely related to serve for definite differentiations. We regarded the injected substances to have acted as antigenic agents in a manner quite similar to egg albumin, milk, fibrinogen solution and the like. Thinking it probable that, while the biochemistry was quite similar in nature, their endotoxin content might present a sufficiently wide range of variance to be of differential value, we accordingly resorted to the Besredka method as modified by Gay.¹

Manner of procedure.—The cultures were grown upon large whiskey flasks containing slanted glycerin agar. After a period of several weeks when the cultures had become quite luxuriant the

¹ Cited by Edith Claypole as a personal communication, *Jour. Exp. Med.*, 1913, 17, p. 108.

TABLE 2.

ANTIGENS	SERA																																	
	Smegma				Kedrowski				Non-Chrome. Duval					Node of Leper					Timothy Hay					Avian Tuberculosis					Chrome. Duval					
	1/40	1/80	1/160	1/320	1/40	1/80	1/160	1/320	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	
Chrome. Duval.	+++	++	+	-	+++	+++	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+++	++	+	-	-	+++	+++	+++	+++	++	
Clegg.....	+++	+++	++	+	+++	+++	++	+	+++	+++	+	-	-	+++	+++	+	-	-	+++	++	++	-	-	+++	++	+	+	-	+++	+++	+++	+++	-	
Kedrowski....	+++	+++	+	-	+++	+++	+	-	+++	+++	+	-	-	+	-	-	-	-	+++	++	++	-	-	+++	+++	+++	+	-	+++	+++	+++	+++	-	
Smegma.....	+++	+++	+	-	+++	+	-	-	++	+	-	-	-	++	+	-	-	-	++	+	-	-	-	+++	+++	++	+	-	+++	++	+	-	-	
Butter.....	+++	+	+	-	-	-	-	-	++	+	-	-	-	++	+	-	-	-	++	+	-	-	-	+++	+++	++	+	-	+++	+++	++	+	-	
Dung.....	+++	+	-	-	-	-	-	-	++	+	-	-	-	++	+	-	-	-	-	+	-	-	-	++	+	-	-	+++	++	++	+	-	-	
Avian T.B.....	+++	+++	+	-	+	-	-	-	++	+	-	-	-	++	+	-	-	-	++	+	-	-	-	+++	+++	++	+	-	+++	++	++	+	-	-
Timothy Hay..	-	-	-	-	-	-	-	-	++	++	-	-	-	++	-	-	-	-	+	-	-	-	-	+++	+	-	-	-	+++	++	+	-	-	

Reagents used: Immune serum, 0.15 c.c.; antigen, 0.5 c.c. of the above dilutions of the 2 per cent stock; complement, 0.5 c.c. of 10 per cent dilution of fresh guinea-pig serum; hemolytic system, 0.5 c.c. of 5 per cent suspension of sheep red blood cells and 2 M.H.D. of specific hemolysin.

Controls: Sera. No hemolytic nor anticomplementary property in double quantities; antigens showed only slight anticomplementary property when double quantities of 1-40 dilution were employed.

growths were scraped from the surface and mixed with saline solution. To these emulsions was added an equal part of absolute ethylic alcohol and after prolonged centrifugalization the supernatant fluid was decanted and the precipitate used. This was desiccated in Novy jars with a partial vacuum over sulfuric acid, and after complete drying they were then weighed and a sufficient amount of sodium chlorid added to constitute 0.85 per cent of the solution when completed. The dried precipitate was ground in combination with the salt in a sterile mortar until an impalpable powder was produced and sufficient sterile water was added to form a 2 per cent solution of the pulverized bacilli. These emulsions were used both for immunizing purposes and to serve as antigens in the tests.

The sera of the rabbits injected intravenously with heavy suspensions of the whole bacilli were tested with the antigens prepared according to the new method and the results are given in Table 2. It must be noted in this table that the rabbits receiving the non-chromogenic strain of Duval and the nodule of a leper were of necessity given injections of much lighter suspensions.

For immunizing with the Besredka antigens two procedures were followed both of which were according to Gay and Fitzgerald.¹ In the first instance, four rabbits were injected intravenously with the following antigen: *B. lepra* (Kedrowski), *B. Smegma*, chromogenic culture (Duval) and the bacillus of timothy hay. They were given three intravenous injections of 0.5 c.c. at 24-hour intervals and were bled the fourth day after the last injection. The results of the sera when tested against the various antigens prepared are tabulated in Table 3.

The second series of rabbits were injected intravenously with the same four acidfast organisms as the previous animals but were given four injections of 0.5 c.c. at three-day intervals and were bled eight days after the last injection. These sera were likewise tested against the antigens and the results are recorded in Table 4.

It will be noted that the anti-substances produced by the three different methods employed show that the whole bacilli emulsion yielded a lower titre than those injected with the Besredka antigen

TABLE 3.

ANTIGENS	SERA															
	Chrome. Duval				Timothy Hay				Smegma				Kedrowski			
	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/400
Chrome. Duval..	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Clegg.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Kedrowski.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Smegma.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Butter.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Dung.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Avian T. B.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Timothy Hay...	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

Reagents used: Immune serum, 0.15 c.c.; antigen, 0.5 c.c. of the above dilutions of the 2 per cent stock; complement, 0.5 c.c. of 10 per cent dilution of fresh guinea-pig serum; hemolytic system, 0.5 c.c. of 5 per cent suspension of sheep red blood cells and 2 M.H.D. of specific hemolysin.

Controls: Sera. No hemolytic nor anticomplementary property in double quantities; antigens showed only slight anticomplementary property when double quantities of 1-40 dilution were employed.

TABLE 4.

ANTIGENS	SERA													
	Chrome. Duval				Timothy Hay				Smegma				Kedrowski	
	1/160	1/320	1/400	1/800	1/160	1/320	1/400	1/800	1/160	1/320	1/400	1/800	1/160	1/320
Chrome. Duval	++	++	+	-	++	++	++	++	++	++	++	++	++	++
Clegg	++	++	++	-	++	++	++	++	++	++	++	++	++	++
Kedrowski	++	++	++	-	++	++	++	++	++	++	++	++	++	++
Smegma	++	++	++	-	++	++	++	++	++	++	++	++	++	++
Butter	++	++	++	-	++	++	++	++	++	++	++	++	++	++
Dung	++	++	++	-	++	++	++	++	++	++	++	++	++	++
Avian T.B.	++	++	-	-	++	++	++	-	++	++	++	++	++	++
Timothy Hay	++	-	-	-	++	++	++	-	++	++	++	++	++	++

Reagents used: Immune serum, 0.15 c.c.; antigen, 0.5 c.c. of the above dilutions of the 2 per cent stock; complement, 0.5 c.c. of 10 per cent dilution of fresh guinea-pig serum; hemolytic system, 0.5 c.c. of 5 per cent suspension of sheep red blood cells and 2 M.H.D. of specific hemolysin.

Controls: Sera. No hemolytic nor anticomplementary property in double quantities; antigens showed only slight anticomplementary property when double quantities of 1-40 dilution were employed.

at 24-hour intervals and that the animals immunized by injections of the latter at three-day intervals and bled after eight days furnish the highest titre of antibodies. The amboceptors in all instances, however, varied only in their degree of titre and showed no specificity either qualitatively or quantitatively. It is not uncommon that the homologous sera and antigen may bind complement at a less high dilution than with a heterogenous antigen.

It would seem that the acidfast organisms as a species may be considered either through their biochemical relationship or through their biological products or both to produce sensibilizators so closely related that little can be expected from this method of procedure for differentiation purposes. Whether from a defense standpoint the bodies produced by the inoculation of either feeble pathogens or saprophytic acidfasts may be of avail in therapeutic lines continues a much discussed problem. Our results have been found quite in accord with those of Gengou,¹ who used likewise a pulverized bacillary antigen prepared by means of dissolving the fat with a potassium hydroxid solution. He employed the following organisms: the homogeneous tubercle bacillus of Arloing, the cold-blooded types of tubercle bacilli of the fish and the blind eel, the acidfast bacillus of horse dung, the butter bacillus (strain, Rabinowitsch-Lille), also the acidfast bacillus of Tobler I, II, V, and the Korn I type and the timothy hay bacillus. Gengou found that these acidfast bacilli, whether of the saprophytic or parasitic varieties, produced, when injected into guinea-pigs, sensibilizators that were active not only for the homologous organism but also for the other members of the group tested. He included in his tests the human, avian, and bovine tubercle bacilli as antigens. His work showed only three exceptions: Tobler I serum did not bind complement in the presence of Tobler V antigen; Tobler II bacilli produced no amboceptor against the fish tuberculosis and Tobler V did not produce any amboceptor against the avian tubercle bacillus. He considered these exceptions of no value and as likely to occur for some of the other organisms used. Koch² found that the serum of animals which had received injections of attenuated

Berl. klin. Wchnschr., 1906, 43, p. 1531.

² Klebs on *Tuberculosis*, 1909, p. 20.

tubercle bacilli contained antibodies common to the bacilli of avian and fish tuberculosis, the hay and the butter bacilli; and conversely, animals treated with the pseudo-tubercle bacilli yielded anti-substances which were common to the true tubercle bacilli. Much,¹ Wills,² and others have likewise found strong relationships of the various members of the acidfast group with the employment of the serum reactions. Claypole³ in her work on the classification of streptothrices included the *B. tuberculosis* (human) and *B. lepra* (chromogenic strain of Duval). She has found no apparent specificity in these instances; for example, the animals immunized against the tubercle bacillus show no binding with 1 to 80 dilution of *B. tuberculosis* antigen whereas partial fixation did occur with *B. lepra* antigen, again a partial fixation at 1 to 40 for *B. tuberculosis* and a complete fixation 1 to 40 for *B. lepra*. Kraus and Hofer⁴ of Vienna, who employed the Pfeiffer phenomena for members of the acidfast group, is of the opinion that specific lysins may be produced if the tests are thus carried on *in vivo*. He feels that he has demonstrated that the chromogenic culture of Duval when injected into guinea-pigs produced a specific lytic substance for the same organism when later injected into the peritoneal cavity, whereas when injecting the lepra culture described by Kedrowski into the Duval animal, such a phenomenon did not occur. He has used this method in an attempt to differentiate the various tubercle bacilli types.

Much,⁵ Deilman,⁶ and others have claimed for this acidfast group the production of three corresponding antibodies: a protein, fatty acids, and a neutral fat (tuberculo-nastin) with which last the most intense fixation occurs. Fitzgerald and Leathes,⁷ however, have shown that pure lipoidal substances are incapable of producing antibodies. Claypole⁸ has suggested that nastin is an impure substance and contains the protein that is characteristic of the acidfast organisms producing fixation with their sera.

¹ *Fortschr. d. Med.*, 1912, 30, pp. 161 and 201.

² *Jour. Exper. Med.*, 1913, 17, p. 99.

³ *Centralbl. f. Bakteriol., Orig.*, 1911, 61, p. 37.

⁴ *Wien. klin. Wchnschr.*, 1912, 25, p. 6.

⁵ *Op. cit.*, and *Beitr. z. Klin. d. Tuberk.*, 1910, 17, p. 199.

⁶ *Ztschr. f. Immunitätsf.*, 1911, 10, p. 421.

⁷ *University of California Publications in Pathology*, 1912, 2, p. 39.

⁸ *Op. cit.*

Whatever may be the facts finally arrived at as to the nature of the anti-substances produced, the results in general of all workers with the complement fixation test in the acidfast group have corresponded, whether the whole bacilli or the various specially prepared antigens were used for immunizing and fixation purposes. The fact that the test serves to differentiate many closely related organisms other than those of the acidfast group would indicate that the acid-resisting bacilli, because of either their chemical analogy or the strong relationship of their intrinsic biological characteristics or both, impede the production of specific amboceptors. It is possible that further refinement of the antigenic preparations with a more detailed chemical analysis of these organisms may permit of specificity production.

CONCLUSIONS.

1. Rabbits injected with whole bacilli or extracts of many of the members of the acidfast group produce anti-substances of a high titre.
2. The whole bacilli produce antibodies of lower potency than that produced by the Besredka antigen. This antigen produces the most potent antibodies when injected intravenously at three-day intervals for four injections and the animal bled after eight days.
3. Regardless of the various methods used to produce these sensibilizators, no clear-cut specificity for complement fixation has been found for the acidfast bacilli made use of in these experiments.

THE TREATMENT OF TETANUS.*

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This research was undertaken with the object of determining as far as is possible, with animals, the relative value of the various methods of treatment of tetanus.

After a trial of a number of laboratory animals, sheep were selected for the reason that they are susceptible to tetanus, large, and easily handled, they could be bled readily, and because it was possible to infect them in a manner approaching clinical conditions. A 14-day broth culture of a virulent strain of tetanus containing large numbers of bacilli and spores was heated at 65° C. for 20 minutes, for the purpose of destroying the toxin.

In order to be sure of this a part of the material was passed through a Berkefeld and then through a Pasteur filter. One-cubic-centimeter doses of the filtrate thus obtained, when injected subcutaneously into 350-gm. guinea-pigs, failed to produce tetanus. Small pieces of wood were macerated in this toxin-free, spore-containing fluid for two weeks, at the end of which time they were removed and dried.

The method of inoculation was as follows. A sheep with a long tail having been selected, an incision one centimeter in length was made through the skin as near the tip as possible. A pocket was now made by blunt dissection. Into this one piece of infected wood was placed and the wound closed by cat-gut sutures. With the onset of symptoms of tetanus, the tail was amputated 20 cm. above the point of inoculation. No anesthetic was used and antiseptics were observed in all operations.

In order to estimate the amount of toxin present in the blood of the sheep two cubic centimeters were removed at 24-hour intervals and injected into the right hind leg of a 350-gm. guinea-pig. In this way a series of animals was obtained which passed from the normal through the various stages of local and general tetanus to

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the death of animals. Thus a reasonably accurate idea of the amount of toxin present in the blood at the time could be obtained.

For the purpose of studying the effects of amputation, without any other treatment, a series of experiments was undertaken by one of us.¹ Fifty guinea-pigs, 30 dogs, and 10 sheep were used. Immediately on the appearance of symptoms the affected members were amputated. No other treatment was used. Among the conclusions drawn, was the following: "Amputation immediately following the first appearance of symptoms affected in no way the progress of the disease. Animals which were amputated died in the same time and with the same symptoms as those which were not amputated."

From these results, it is reasonable to conclude, therefore, that any benefit obtained by treatment is the result of this treatment and not of amputation.

Having established this point, we undertook the study of the various methods of treatment, as follows: antitetanic serum, carbolic acid method of Bacelli, magnesium sulfate, chlorbutanol alone and in combination with serum.

Six sheep were used in each series, five being treated while one was used as control. In every series, the control animal died with practically identical symptoms in each case.

TABLE 1.
CONTROL SHEEP.

Sheep No.	Series	Inoculated	Symptoms	Amputated	Results
14.....	Carbolic acid	Oct. 29	Nov. 5	Nov. 5	Died Nov. 11
112.....	Magnesium sulfate	March 26	April 4	April 4	" April 6
301.....	Chlorbutanol	July 1	July 8	July 8	" July 12
163.....	Chlorbutanol and serum	" 13	" 20	" 20	" " 24
274.....	Serum	Dec. 13	Dec. 13	Dec. 13	" Dec. 15

A SAMPLE PROTOCOL.

Sheep 14, weight 32.6 kilos, control, inoculated as described on October 29, 1909. Sheep appeared normal until 7 days later, when there was noticed a slight stiffening of the front legs when the sheep attempted to run. The neck was held rigid with the nose slightly extended. Eating and drinking were normal. The tail was at once amputated 20 cm. above point of inoculation under antiseptic precautions and the wound closed with cat-gut. Healing of the wound was uneventful.

¹ Hutchings, *Rindfleisch-Festschrift*, p. 601, Leipzig, 1907.

Eighth day: Symptoms were more marked; the sheep walked with some difficulty and fell to knees when attempting to run. Neck stiffer, ate and drank well, chewed cud, and had no difficulty in swallowing.

Ninth day: The sheep remained lying down, but could get up with difficulty. Stiffness pronounced. Ate and drank little. Did not chew cud.

Tenth day: Could not get up alone and when placed on feet could take only a few steps without falling. Ate nothing and swallowed water with difficulty. Stiffness of whole body pronounced.

Eleventh day: Lying on side with head hyper-extended, legs extended and perfectly rigid, could not stand.

Twelfth day: About the same as on the previous day; a few clonic spasms were seen.

Thirteenth day: Clonic spasms frequent but not severe, lasting about 30 seconds. Died during the afternoon.

The following table shows the result of the injection of this sheep's blood into the guinea-pigs.

TABLE 2.

Pig	Weight gm.	Inoculated with 2 c.c. on	Symptoms	Date	Result
1.....	250	Oct. 30	—		Recovered
2.....	305	" 31	—		"
3.....	275	Nov. 1	+	Nov. 5	"
4.....	290	" 2	+	" 5	"
5.....	308	" 3	+	" 5	Died
6.....	310	" 4	+	" 6	"
7.....	315	" 5	+	" 6	"
8.....	315	" 6	+	" 7	Recovered
9.....	305	" 7	+	" 10	"
10.....	335	" 8	+	" 10	"
11.....	280	" 9	+	" 11	"
12.....	325	" 10	+	" 13	"
13.....	330	" 11	+	" 14	"

From Table 2 it will be seen that measurable amounts of toxin appeared in the blood on the third day, that the toxin was at its maximum from the fifth to the eighth, and persisted until death on the thirteenth day, but that after the eighth day the amount present was small. On the other hand, the clinical symptoms were not discoverable until the seventh day and reached what might be termed a maximum on the eleventh day. Had this train of symptoms appeared in the ordinary case of tetanus in human beings it is probable that the patient would not have come under observation before the eighth day and a diagnosis would not have been reached until the ninth or even the tenth day. From the table one would be inclined to believe that the decrease of toxin in the circulation after the eighth day was a result of the amputation, removal of the source of supply, but that this is not true we have been repeatedly

shown. We have found that the amount of demonstrable toxin in the blood of animals having tetanus, with and without amputation, runs a course practically identical with that shown in the table.

SERUM.

Six sheep were used, five treated with intravenous injection of 2,000 units of antitetanic serum every 24 hours, one control. The control died (274). Of those treated four died and one recovered. The protocol of the one that recovered is as follows:

Sheep 108, weight 55.5 kilos.

December 6: Inoculated in tail as usual.

Seventh day: Showed marked stiffness of head and neck. Some stiffness in forelegs, otherwise normal. Tail amputated 20 cm. above point of inoculation. 2,000 units serum injected into left jugular vein.

Eighth day: Somewhat stiffer than day before. Walked with difficulty. While being prepared for bleeding and injection, had several slight clonic spasms and a peculiar "thrill" which is characteristic of tetanus in guinea-pigs. Ate and drank little. 2,000 units serum injected into left jugular vein.

Ninth day: No worse than on previous day. 2,000 units serum injected.

Tenth day: About the same except that he ate and drank better. 2,000 units serum injected.

Eleventh to sixteenth day: Condition remained stationary. 2,000 units of serum were given on each of these days when its use was discontinued. The sheep developed chronic tetanus and was not completely recovered until February 1.

The total amount of serum given was 20,000 units.

TABLE 3.
RESULTS OF INOCULATION WITH BLOOD OF SHEEP 108.

Pig	Weight gm.	Inoculated with 2 c.c. on	Symptoms	Date	Result
1.....	350	Dec. 6	—		
2.....	"	" 7	—		
3.....	"	" 8	—		
4.....	"	" 9	—		
5.....	"	" 10	+	Dec. 13	Recovered
6.....	"	" 11	+	" 13	Died
7.....	"	" 12	+	" 14	"
8.....	"	" 13	+	" 16	Recovered
9.....	"	" 14	—		
10.....	"	" 15	—		
11.....	"	" 16	—		
12.....	"	" 17	—		
13.....	"	" 18	—		

The protocol of the sheep which died follows:

Sheep 277, weight 40 kilos.

December 6: Inoculated as usual.

Eighth day: Showed slight stiffness in forelegs, head was slightly extended, otherwise normal. Tail amputated. 2,000 units of serum injected intravenously.

Ninth day: Stiffness increased, drank well but ate little. 2,000 units of serum injected intravenously.

Tenth day: Stiffness very marked, unable to walk but could stand. Refused all food and could not swallow water. Rapid breathing. 2,000 units of serum injected intravenously.

Eleventh day: Very little change from previous day. 2,000 units of serum injected intravenously. Died during the night.

TABLE 4.
RESULTS OF INJECTIONS WITH BLOOD FROM SHEEP 277.

Pig	Weight gm.	Inoculated with 2 c.c. on	Symptoms	Date	Results
1	320	Dec. 5	—		
2	300	" 7	—		
3	335	" 8	—		
4	300	" 9	—		
5	250	" 10	+	Dec. 14	Recovered
6	255	" 11	+	" 13	Died of tetanus
7	330	" 12	+	" 14	" Dec. 17
8	330	" 13	+	" 14	" " 18
9	325	" 14	+	" 15	" " 18
10	325	" 15	—		
11	325	" 16	—		
12	320	" 17	—		

TABLE 5.
SHEEP TREATED WITH SERUM ALONE.

Sheep	Inoculated	Symptoms	Amputated	Treatment 2,000 Units	Results
108	Dec. 6	Dec. 13	Dec. 13	Serum daily	Recovered
277	" 6	" 14	" 14	" "	Died Dec. 18
12	" 6	" 12	" 12	" "	" " 18
275	" 6	" 14	" 14	" "	" " 18
299	" 6	" 14	" 14	" "	" " 22

OBSERVATION ON SERUM SHEEP.

1. Twenty-four hours after the first injection of serum, no toxin was found in the blood. This was true of all five sheep.

2. In four of the five sheep, despite the absence of toxin in the blood, the symptoms were not influenced.

CARBOLIC ACID.

Before discussing the experiments with sheep it may be of interest to give a few of the preliminary experiments with guinea-pigs. We tried first to determine how much carbolic acid was necessary to kill an average pig.

Series 1.—0.5 c.c. of a two per cent solution of carbolic acid in water was injected every two hours until 32 doses were given, without producing any effect. The amount was then increased to one cubic centimeter every two hours. Of this, 11 doses were given before the animals showed symptoms of poisoning.

Series 2.—Two other pigs weighing 275 and 256 gm. respectively were given 0.5 c.c. of a two per cent solution until 42 doses were given without producing any effect. Treatment was then discontinued. Ten days later the pigs were alive and in good condition.

Series 3.—Five pigs were given 10×M.F.D. of U.S. government standard tetanus toxin. With the first appearance of symptoms they were given 0.5 c.c. of a two per cent carbolic solution every two hours during the day without any apparent effect. All died.

Series 4.—Five pigs each received 10×M.F.D. tetanus toxin. The carbolic acid was given every two hours day and night with the result that two pigs recovered and two died.

Series 5.—Carbolic injections were begun with two of the pigs immediately after the injection of the toxin with the purpose of determining whether it would prevent the development of tetanus. This it failed to do as the pigs developed the disease and died.

Sheep treated with carbolic acid.—Six sheep were used, one as control. Of the sheep treated all received two cubic centimeters of a two per cent carbolic solution every two hours day and night. All died. In comparison with the sheep in which serum alone was used, there was a very slight difference in the tonic muscular contractions, the carbolic sheep being less stiff. The difference, however, was not as marked as with magnesium sulfate and chlorbutanol. It had no influence on the course of the disease.

The following is a sample protocol:

Sheep 17, weight 44.5 kilos.

October 29: Inoculated in the usual way.

Eighth day: Showed stiffness in the front legs, head extended. Stump of tail extended. (This was the only sheep which showed any local tetanus.) Tail amputated 20 cm. above point of inoculation. Two cubic centimeters of a two per cent carbolic solution injected subcutaneously every two hours.

Ninth day: Symptoms more marked. Impossible to get up, but could stand when placed on feet; could not walk without falling. Could not swallow.

Tenth day: Very little change from previous day.

Eleventh day: 10 A.M. clonic convulsions. Died at 8 P.M.

MAGNESIUM SULFATE.

Our results with magnesium sulfate were unsatisfactory, all of the animals dying either as a result of tetanus or as a result of magnesium sulfate poisoning. Six sheep were used, the control (No. 112) dying of tetanus. Of the five sheep treated, two were given 1.25 gm. per kilo body weight; three were given 2.50 gm. Those receiving the smaller amount showed no effect, while the latter all died apparently of magnesium sulfate poisoning.

PROTOCOLS.

Sheep 87, weight 35 kilos.

March 26. Inoculated.

Ninth day, 8:30 A.M.: Sheep slightly stiff. Tail amputated, 1.25 gm. magnesium sulfate in salt solution per kilo injected subcutaneously into abdominal wall under aseptic precautions. Fifteen minutes required for injection. During the day the sheep showed no signs of relaxation but became gradually stiffer.

Tenth day: Sheep had well marked tetanus. Unable to get up or stand when placed on feet. Magnesium sulfate repeated, but produced no effect. During the day the sheep became very stiff, legs and neck hyper-extended.

Eleventh day: Dead in stall.

TABLE 6.

Pig	Weight gm.	Inoculated with 2 c.c. on	Symptoms	Date	Result
1.....	350	March 26	—		
2.....	"	" 27	—		
3.....	"	" 28	—		
4.....	"	" 29	+	April 1	Recovered
5.....	"	" 30	+	" 1	"
6.....	"	" 31	+	" 1	"
7.....	"	April 1	+	" 2	Died
8.....	"	" 2	+	" 3	"
9.....	"	" 3	+	" 4	"
10.....	"	" 4	+	" 5	"
11.....	"	" 5	+	" 6	Recovered

Protocol of sheep which died from magnesium sulfate poisoning:

Sheep 268, weight 32 kilos.

March 26. Inoculated.

Eighth day: Sheep showed slight signs of stiffness but moved about without difficulty. Tail amputated. 2.5 gm. per kilo of body weight of magnesium sulfate in salt solution injected subcutaneously into abdominal wall under aseptic precautions. At 12:00 the sheep was lying on its side in the stall completely anesthetized. There was relaxation of all muscles. When raised from the floor the animal was quite limp. There was a considerable amount of froth around the mouth and mucous exudate from the nose. Respirations were shallow and infrequent, 12 per minute. This condition was unchanged at 5 P.M.

Ninth day: Sheep dead in stall.

TABLE 7.

Pig	Weight gm.	Inoculated with 2 c.c. on	Symptoms	Date	Result
3	350	March 26	—		
1	"	" 27	—		
7	"	" 28	—		
4	"	" 29	+	April 2	Recovered
5	"	" 30	+	" 2	"
0	"	" 31	+	" 3	Died
7	"	April 1	+	" 3	"
8	"	" 2	—	" 3	"
9	"	" 3	—	" 4	Recovered

CHLORBUTANOL.

In administering chlorbutanol to sheep we met with many difficulties. The drug is very slightly soluble in water, 0.8 of one per cent. and somewhat less in normal salt solution. For this reason it was impossible, on account of the amount required, to give it subcutaneously or intravenously. A stomach tube can be passed with little difficulty on a sheep which does not have tetanus. In this way sufficient chlorbutanol can be given in a dilute alcoholic solution. However, when the animal has tetanus, the passage of a stomach tube frequently causes prolonged contraction of the muscles of respiration, leading to death. In our preliminary work several were killed in this way. Chlorbutanol is readily soluble in hot olive oil and remains in solution when the oil is cool. Taking advantage of this fact, we injected chlorbutanol dissolved in oil intraperitoneally. By this method the animals were affected almost as quickly as when the drug was given per stomach. Unfortunately, however, the repeated punctures necessary in a prolonged treatment in many cases cause peritonitis. Altho conducted under aseptic precaution it was not always possible to avoid perforating the gut. In one series we treated the infected animals with chlorbutanol alone and in another with chlorbutanol in combination with serum. The series treated with chlorbutanol alone is as follows:

Six sheep were used, one as control, No. 301. Of these, two died of peritonitis due to the perforation by the injecting needle. One died probably of chlorbutanol poisoning. Two recovered.

The protocols of those dying of peritonitis are not instructive beyond the fact that the muscular symptoms of the tetanus were

entirely controlled and the amount of toxin present in the blood was not affected.

The protocol of the sheep dying of chlorbutanol poisoning is as follows:

Sheep 46, weight 36 kilos.

July 1. Inoculated in usual way.

Seventh day: Sheep slightly stiff, particularly the neck, which he seemed disinclined to move. Tail amputated. Seven grams of chlorbutanol in 100 c.c. of olive oil injected intraperitoneally. One-half hour after injection the sheep was lying on its side apparently asleep. Reflexes, however, were present. Pressure in amputation stump caused slight movement.

Eighth day: In same position and apparently in same condition as day before. Unable to stand and refused to drink.

Ninth day: Sheep lying down but could be easily aroused. Drank a little water with an effort probably due to position. During the day the sheep became wider awake and showed stiffness of forelegs and neck.

5 P.M.: Intraperitoneal injection of 7 grams of chlorbutanol in 100 c.c. of oil.

5:30 P.M.: The animal was completely anesthetized. No eye reflex.

Tenth day: Sheep found in same position as left the night before and dead. Death probably due to chlorbutanol poisoning.

The guinea-pigs injected with blood from this sheep showed the usual results.

The protocols of the two sheep recovering were similar.

Sheep 37, weight 34 kilos.

Inoculated July 1.

Seventh day: Sheep quite stiff. Tail amputated. Intraperitoneal injection of four grams of chlorbutanol in 100 c.c. of olive oil. One hour later the sheep was lying on one side asleep but could be easily aroused. All rigidity of muscles absent. Remained this way during the day.

Eighth day: Sheep lying down but aroused easily. Drank about one-half liter of water. Refused to eat. Four grams of chlorbutanol given intraperitoneally. Sheep remained sleeping during the day.

Ninth day: Sheep in same condition as previous day, not disturbed. No chlorbutanol.

Tenth day: Not moved from position in which he was placed previous day. Drank a small amount of water. No chlorbutanol.

Eleventh day: Sheep awake but unwilling to move. Refused food but drank freely about 1,500 c.c. of water. During the afternoon showed slight return to stiffness.

Twelfth day: Stiffness more marked. Drank water but refused to eat. Four grams of chlorbutanol injected intraperitoneally.

Thirteenth to fifteenth day: Sheep remained quietly sleeping. On the fourteenth day drank a little water. On the morning of the fifteenth day was awake and apparently hungry but could not swallow food. There was no sign of muscular rigidity.

From this time the animal continued to drink and on July 17 ate well. There was no sign of the tetanus from this time.

Pigs injected are as follows:

TABLE 8.
RESULTS OF INOCULATION WITH BLOOD OF SHEEP 37.

Pig	Weight	Inoculated with 2 c.c. on	Symptoms	Date	Result
1	350	July 1	—		
2	"	" 2	—		
3	"	" 3	—		
4	"	" 4	+	July 7	Recovered
5	"	" 5	+	" 7	"
6	"	" 6	+	" 8	"
7	"	" 7	+	" 8	Died
8	"	" 8	+	" 9	"
9	"	" 9	+	" 10	Recovered
10	"	" 10	+	" 11	"
11	"	" 11	+	" 12	"
12	"	" 12	+	" 14	"
13	"	" 13	+	" 15	"
14	"	" 14	+	" 16	"
15	"	" 15	+	" 17	"
16	"	" 16	+	" 19	"
17	"	" 17	—		
18	"	" 18	—		
19	"	" 19	—		
20	"	" 20	—		

CHLORBUTANOL AND SERUM.

Six sheep were used, one as control, No. 163. Of the five treated one died of peritonitis due to perforation. Four recovered.

The protocol of the sheep which died is as follows:

Sheep 300, weight 33.5 kilos.

Inoculated as usual July 13.

Seventh day: Showed slight stiffness in the forelegs. Tail amputated. Five grams of chlorbutanol in 100 c.c. of olive oil injected intraperitoneally and 2,000 units of serum injected intravenously. During the injection the sheep got away from the

TABLE 9.
RESULTS OF INOCULATION WITH BLOOD OF SHEEP 300.

Pig	Weight	Inoculated with 2 c.c. on	Symptoms	Date	Result
1	350	July 13	—		
2	"	" 14	—		
3	"	" 15	—		
4	"	" 16	+	July 18	Recovered
5	"	" 17	+	" 19	"
6	"	" 18	+	" 19	Died
7	"	" 19	+	" 20	"
8	"	" 20	+	" 22	Recovered
9	"	" 21	—		

attendant and was kept on the table with difficulty. One hour after the injection the sheep was lying on its side asleep. During the afternoon it remained quiet but at 5:30 P.M. abdomen was distended.

Eighth day: Sheep remained quiet but abdomen was greatly distended. 2,000 units of serum injected.

Ninth day: Sheep dead in stall.

Autopsy showed three perforations of the gut, and in the exudate a streptococcus and a long bacillus were found.

TABLE 10.
RESULTS OF INOCULATIONS WITH BLOOD OF SHEEP 110.

Pig	Weight	Inoculated with 2 c.c. on	Symptoms	Date	Result
1.	350	July 13	—		
2.	"	" 14	—		
3.	"	" 15	—		
4.	"	" 16	+	July 18	Recovered
5.	"	" 17	+	" 18	"
6.	"	" 18	+	" 19	Died
7.	"	" 19	+	" 20	"
8.	"	" 20	+	" 21	"
9.	"	" 21	—		Recovered
10.	"	" 22	—		"
11.	"	" 23	—		"
12.	"	" 24	—		"
13.	"	" 25	—		"
14.	"	" 26	—		"
15.	"	" 27	—		"
16.	"	" 28	—		"
17.	"	" 29	—		"
18.	"	" 30	—		"
19.	"	Aug. 1	—		"
20.	"	" 5	—		"

The following is a protocol of one of the four sheep which recovered:

Sheep 110, weight 36 kilos.

Inoculated as usual on July 13.

Seventh day: Sheep slightly stiff in forelegs and neck. Tail amputated. Five grams of chlorbutanol injected intraperitoneally and 2,000 units of serum intravenously. One hour later sheep was lying quietly on its side asleep, relaxation complete.

Eighth day: In the same position as previous day, completely relaxed. 2,000 units of serum injected. During the day sheep remained quiet but at 5:30 P.M. began to wake up but refused to drink.

Ninth day: Sheep well awake and slightly stiff. Drank about one liter of water but refused food. Five grams of chlorbutanol in 100 c.c. of olive oil injected intraperitoneally and 2,000 units of serum intravenously. Went to sleep immediately and remained so during the entire day.

Tenth day: In same position as previous day and asleep. No stiffness. 2,000 units of serum injected intravenously.

Eleventh day: Remained lying on side during the day but could be aroused and at 5:30 P.M. drank a little water. 2,000 units of serum. No chlorbutanol.

Twelfth day: Sheep awake and slightly stiff. 2,000 units of serum injected intravenously. During the day the sheep tried to get up but was unable to because of stiffness which had increased to a considerable extent.

5 P.M.: Intraperitoneal injection of 5 grams of chlorbutanol.

Thirteenth to fifteenth day: Sheep remained sleeping quietly. At 5:30 P.M., July 27, could be aroused and drank about 1,500 c.c. of water. On each of these days 2,000 units of serum were given intravenously.

Sixteenth day: Sheep awake but not stiff. During the day remained drowsy but the stiffness did not return as on previous days.

Seventeenth day: Sheep awake, could stand when placed on feet but was very weak. Drank well and ate a small amount.

Eighteenth day: Improved in all conditions. No stiffness and walked a little. Sheep rapidly recovered strength and on August 5 was in normal condition.

The pigs injected were as given in table 10.

CONCLUSIONS.

It appears to us that the following conclusions are warranted by these experiments.

1. Amputation after the appearance of symptoms is of no value.
2. The toxin appearing in the blood stream is self-limited even in the fatal cases.
3. There is little if any value in the carbolic acid treatment of the disease.

If there is any gain, our opinion is that it is probably due to the sedative action of the drug and not to any direct action on the disease process, and that this result may be obtained with greater certainty by other drugs.

4. The magnesium sulfate as used in our experiments, subcutaneously, is of no value.

5. Antitetanic serum alone has a definite, altho usually insufficient curative effect.

6. It appears to us, from the observation of a large number of animals and quite a number of human beings dying of tetanus, that the exhaustion due to the muscular contractions is a large factor in producing fatal results. For this reason much of our work has been given to the attempt to hold these convulsions in check.

7. The presence of a large amount of toxin in the blood several days (in sheep it can be demonstrated four days) before the onset of clinical symptoms makes it imperative that a method be devised for easily determining this. With such a method it is quite probable that we could save a large proportion of our tetanus cases.

8. With our present knowledge of the subject, the best that can be done in the treatment of tetanus is to neutralize the toxin with repeated doses of serum while controlling the muscular spasm with some such drug as chlorbutanol.

A STUDY OF THE TYPHOID COLON INTERMEDIATE GROUP OF BACILLI, WITH SPECIAL REFERENCE TO COMPLEMENT-FIXATION REACTIONS.*

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One of the most difficult problems in bacteriology is the study, classification, and differentiation of members of a "group" of closely allied organisms. Thus individual members may differ from each other in pathogenicity but possess such similar morphological characteristics as to require most careful biological and cultural study for differentiation. Frequent and persistent efforts have been made in attempts to transform types and while the secondary and non-essential features may be changed temporarily, such as dimensions, motility, growth upon ordinary culture media, etc., the cardinal and vital characteristics remain on the whole unchanged. For purposes of study and classification a prominent member is selected as the characteristic organism of the group and the remaining members grouped under that head. Owing partly to the fact that the various organisms of a "group" are found living in the same portions of the body as the diphtheria group in the upper air passages, the colon-typhoid group in the intestinal tract, etc., it is widely accepted that the various members of a group may have been derived from an original species, but owing to long-continued variations in environment have adopted slight but fairly well-defined cultural and biological characteristics which are transmitted from generation to generation. This theory of "mutation" has much in its support as relates to bacteria differing but slightly from the parent, but as regards the relationship between the more prominent members of a group it is hardly tenable. Thus much work has been reported upon the relation of *B. coli* to *B. typhosus* and the possibility that *B. typhosus* may have been derived

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originally from *B. coli* by a loss of certain zymogenic and the acquisition of pathogenic properties, but the possibility of this change is not proven by experimental investigation, notwithstanding various reports concerning changes in cardinal characteristics, as *B. coli* losing its power of producing indol and coagulating milk or *B. typhosus* producing indol upon prolonged cultivation.

Most reliance in the differentiation of members of a group of closely allied organisms is placed upon their fermentative action upon carbohydrate media, agglutination reactions, and tests for pathogenicity and virulence. Since the discovery by Bordet and Gengou of the specific relationship of antigen and antibody as determined by complement-fixation experiments, this method of investigation has been applied to the study of "groups" of organisms based upon the assumption of strict specificity of antigen for its immune amboceptor. Thus Besredka,¹ Foix and Mallein,² Swift and Thro³ have reported that immune bodies specific for different strains of streptococci can be demonstrated by means of the complement-fixation test. Kolmer⁴ working with five strains of streptococci, with the specific purpose of studying the relationship of the streptococcus commonly found associated with scarlet fever to the group of streptococci, found that differentiation among these was possible when using high dilutions of the immune sera but in lower dilutions differentiation was not found by means of complement-fixation tests, the results indicating either that this scarlet-fever streptococcus belonged to the common group of streptococci or that the complement-fixation test was but a group reaction. In a similar study applied to the diphtheria group of bacilli with the purpose of studying the relationship of Hofmann's bacillus or the pseudo-diphtheria bacillus to the true virulent Klebs-Loeffler organism, Kolmer⁵ found analogous results, namely, that the pseudo-diphtheria bacillus could not be differentiated from other members of the group by complement-fixation reactions, indicating that this bacillus was probably derived originally from the parent of the group and has been gradually so changed by environment as to

¹ *Ann. de l'Inst. Pasteur*, 1904, 28, p. 363.

² *Presse med.*, 1907, 15, p. 777.

³ *Arch. Int. Med.*, 1911, 7, p. 24.

⁴ *Ibid.* 1912, 9, p. 220.

⁵ *Jour. Infect. Dis.*, 1912, 11, p. 44.

adopt certain cultural and transmissible characteristics, thus constituting another example of "mutation" among bacteria.

In this communication is reported a similar study applied to the typhoid-colon group of bacilli. While it is comparatively easy to differentiate the typhoid from the colon bacillus, there is an intermediate group between the two which partakes of the morphological, cultural, and pathogenic properties of both, rendering their practical differentiation extremely difficult. The bacilli of this "intermediate group" have attracted attention chiefly in connection with meat poisoning, protracted fevers clinically indistinguishable from mild typhoid infections, and in certain acute intestinal infections of the lower animals. Attempts to study the interrelationship of various members and systematize their grouping have been made by Buxton, Durham, Kutscher and Meinicke, and many others with special reference to cultural characteristics, fermentative action upon carbohydrate media, and agglutination reactions with immune sera, and the latter two methods of study probably remain the best means of differentiation.

In a general way the object of this study was to further investigate the specificity of complement-fixation reactions in the differentiation of bacteria of a group. Since members of a group bear such a close cultural and morphological relationship to each other, it must be expected that the antibodies, as agglutinins and amboceptors, would not be absolutely specific for their antigen or if the amboceptor were specific it would demonstrate this specificity only with an antigenic substance not common to the group. In such a study, therefore, the question of preparation of antigen becomes quite important.

Thus the purpose of the investigation was:

(1) To make a further study of complement-fixation reactions in the differentiation of bacteria belonging to a general "group."

(2) To determine more specifically if such reactions would demonstrate the relationship of the members of the typhoid-colon group of bacilli to each other and serve as a means of differentiation among them.

(3) To compare the efficiency of complement-fixation reactions

with acid and gas production tests and agglutination reactions in differentiating among the members of the group.

METHOD OF STUDY.

This consisted in the careful selection from a number of cultures of 14 strains of bacilli belonging to the typhoid-colon group.¹ These were studied for gas and acid production with various sugars in litmus and neutral red broth; for indol production, coagulation of milk, etc. Rabbits were immunized and the sera used for agglutination and complement-fixation experiments.

1. *Cultures*.—The following cultures were selected for the study from a large number at our disposal:

No.	Organisms	
1	<i>B. coli communior</i>	Coli group
2	<i>B. coli communis</i>	
3	<i>B. lactis aërogenes</i>	
4	<i>B. enteritidis</i>	
5	<i>B. paracoli</i>	Enteritidis group
6	<i>B. hog cholera</i>	
7	<i>B. fecalis alcaligenes</i>	
8	<i>B. paratyphosus A</i>	
9	<i>B. paratyphosus A</i>	Typhosus group
10	<i>B. paratyphosus B</i>	
11	<i>B. dysenterica (Flexner)</i>	
12	<i>B. typhosus, Strain A</i>	
13	<i>B. typhosus, Strain B</i>	
14	<i>B. cholera</i>	

Each culture has been given a number and the immune serum and "antigen" of a culture is designated according to the number in recording the agglutination and complement-fixation experiments. Thus No. 1 refers to culture of *B. coli communior*, its immune serum, antigen, etc.

The cultures have been divided into groups according to Park, except that *B. dysenterica* is placed in the typhosus group. This arrangement is based upon the principal or cardinal characteristics of closely allied species and has proven of much aid in a study of the entire group.

a) *Coli group*.—Dextrose and lactose fermented with gas formation; milk coagulated with acid production; motility sluggish; indol produced by most varieties.

b) *Enteritidis group*.—Dextrose fermented with gas formation;

¹ We wish to express our thanks to Dr. K. F. Meyer, Dr. D. Rivas, Dr. John Reichel, and the Museum of Natural History, New York, for many cultures kindly given us.

lactose not fermented; milk not coagulated; no indol or only slight amounts produced; motility usually marked (*B. alcaligenes* ferments no sugars).

c) *Typhosus* group.—Dextrose fermented without gas production; lactose not fermented; milk not coagulated; no indol except with certain types of *B. dysenterica*; milk not coagulated; *B. dysenterica* non-motile.

While *B. cholera* does not belong to the group of typhoid-colon bacilli, it was included in this study as a control.

Our aim was to select representative cultures as different cultures of the same species will show minor variations. Thus all cultures of *B. coli* possess major characteristics, as acid and gas productions with glucose, indol formation, coagulation of milk, etc., but cultures possessing these cardinal characters will differ from each other in their action with other sugars, as saccharose, raffinose, and mannite. In the face of this variability of *B. coli* it is to be expected that minor variations occur with different cultures of *B. typhosus*, not to mention the possible variations among the intermediates. Fermentation reactions are usually chosen as the means of classification, because of the comparative ease with which the organisms may be differentiated from those of other groups and because of the facility with which each variety may be separated from the others. The characteristics of the cultures used in this study are given in Table 1.

The differentiation between *B. coli communior* and *B. coli communis* is based upon the investigations of Durham, the former producing gas with saccharose as well as with dextrose and lactose, the latter only with dextrose and lactose. According to the work of Jackson¹ the culture of *B. coli communior* belongs to type A and *B. coli communis* to type B.

It will be noted that with trypsinized broth (Rivas²), indol could be demonstrated in 24 hours with the cultures of *B. coli*. A parallel series were conducted with peptone (Witte) water and tested after 12 days' incubation. The results were similar with the exception of *B. lactis aërogenes* which produced indol in peptone water but not within 24 hours in the trypsinized broth. In using the latter

¹ Jour. Amer. Pub. Health Assoc., 1911, 1, p. 930.

² Centralbl. f. Bakteriöl., I Orig., 1912, 63, p. 547.

medium care must be taken not to carry the process of digestion too far and controls should be used for the same reason as testing for indol with peptone water. With these precautions the trypsinized broth gives excellent results.

All cultures were tested with the various sugars for acid production or reduction of neutral red broth. With this medium the presence of acid may be shown in two different ways: (a) the neutral red broth becomes dark purple in color or (b) the broth is decolorized or of a yellowish color. In both instances the changes are due to the presence of acid but just why the different colors are

TABLE 1.

No.	ORGANISM	INDOL		Milk	GELATIN	UCHINSKY	MOTILITY	SUGARS											
		Tryp- sin- ized Broth 24 hrs.	Peptone 12 days					Dulcitol	Dextrose	Galactose	Levulose	Maltose	Lactose	Saccharose	Raffinose	Inulin	Dextrin	Mannite	Arabinose
1	B. coli communior.	—	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	B. coli communis.	—	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	B. lactis aerogenes.	—	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	B. enteritidis.	—	—	×	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	B. paracoli.	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	B. bog cholera.	—	—	×	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	B. fecalis alcaligenes.	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	B. paratyphosus A.	—	—	×	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	B. paratyphosus A.	—	—	×	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	B. paratyphosus B.	—	—	×	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	B. dysenterica Flexner.	+	+	×	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	B. typhosus, Strain A.	—	—	×	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	B. typhosus, Strain B.	—	—	×	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+

— Acid and gas (sugars), acid and clot (milk).

× Acid without gas (sugars), or acid without clot (milk).

= Doubtful.

produced we are unable at present to say, unless the changes are due to the formation of different acids or of one acid in excess with the different sugars. The results of these tests are given in Table 2. Cultures were incubated 48 hours at 37° C. and the results recorded are those occurring in this time and not after a more prolonged period of incubation.

By comparing acid production in litmus and neutral red broth in Tables 8 and 9 it will be noted that different results occurred, especially with the three sugars: levulose, maltose, and lactose. In both sets of experiments the results recorded are those occurring after 48 hours' incubation. These differences may be tabulated as in Table 3.

Whether or not these differences are due to the different action of the acids produced upon the indicator or to the question of time of incubation, etc., is being investigated with the larger number of cultures.

TABLE 2.
ACID PRODUCTION IN NEUTRAL RED BROTH.

No.	ORGANISM	NEUTRAL RED BROTH											
		Dulcite	Dextrose	Galactose	Levulose	Maltose	Lactose	Saccharose	Raffinose	Inulin	Dextrin	Mannite	Arabinose
1	<i>B. coli communior</i>	+	+	+	-	×	-	×	×	-	×	+	×
2	<i>B. coli communis</i>	+	+	+	-	×	-	+	×	-	×	+	×
3	<i>B. lactis aërogenes</i>	+	+	+	×	×	×	+	×	-	×	×	×
4	<i>B. enteritidis</i>	+	+	+	-	-	-	-	×	-	-	+	+
5	<i>B. paracoli</i>	+	+	+	-	-	-	-	×	-	+	+	+
6	<i>B. hog cholera</i>	-	+	+	-	-	-	-	+	-	+	+	+
7	<i>B. fecalis alcaligenes</i>	-	+	-	-	-	-	-	+	-	+	×	×
8	<i>B. paratyphosus A</i>	-	+	+	-	-	+	-	-	-	+	+	+
9	<i>B. paratyphosus A</i>	-	+	+	-	-	-	-	-	-	+	+	+
10	<i>B. paratyphosus B</i>	-	+	+	-	-	+	-	+	-	-	×	+
11	<i>B. dysenterica</i> (Flexner)	-	+	+	-	-	+	-	+	-	-	+	+
12	<i>B. typhosus</i> , Strain A	-	-	+	-	-	+	-	-	-	-	+	+
13	<i>B. typhosus</i> , Strain B	-	-	+	-	-	+	-	-	-	-	+	+

+ Reduction (acid) with purplish color.

× Reduction (acid) with yellow color.

= Doubtful.

TABLE 3.
DIFFERENCES IN ACID PRODUCTION BETWEEN LITMUS BROTH AND NEUTRAL RED BROTH.

No.	ORGANISM	LITMUS BROTH			NEUTRAL RED BROTH		
		Levulose	Maltose	Lactose	Levulose	Maltose	Lactose
1	<i>B. coli communior</i>	+	+	+	-	+	-
2	<i>B. coli communis</i>	+	+	-	-	+	+
3	<i>B. aërogenes</i>	+	+	+	+	+	+
4	<i>B. enteritidis</i>	+	+	-	-	-	-
5	<i>B. paracoli</i>	+	+	+	-	-	-
6	<i>B. hog cholera</i>	+	+	-	-	-	-
7	<i>B. alcaligenes</i>	-	-	-	-	-	-
8	<i>B. paratyphosus A</i>	+	+	-	-	-	+
9	<i>B. paratyphosus A</i>	+	+	-	-	-	-
10	<i>B. paratyphosus B</i>	+	+	-	-	-	+
11	<i>B. dysenterica (Flexner)</i>	+	+	+	-	-	+
12	<i>B. typhosus</i>	+	+	+	-	-	+
13	<i>B. typhosus</i>	+	+	+	-	-	+

2. *Immune sera*.—These were prepared by giving a series of rabbits intravenous injections of increasing doses of pure cultures of bacilli suspended in salt solution and heated to 58° C. for one hour, until a full set of highly agglutinative sera were secured.

The sera of a number of typhoid fever convalescents were also

used in a brief study concerning the practical application of complement-fixation reactions to the diagnosis of infection with a member of the group.

AGGLUTINATION REACTIONS.

The value of agglutination reactions in differentiating among the members of this group of bacilli is too well known for further comment. Our object was to compare the specificity of the agglutinins and the amboceptors by a series of cross-agglutination experiments. Thus the immune serum of one member of the group is tested against all cultures to determine its agglutinin content for the allied bacilli as a means for studying their relationship and for comparison with the complement-fixation reactions.

Method.—The macroscopic technic was employed. Cultures were grown in neutral broth, filtered and used in doses of 1 c.c. The serum dilutions were made up to 1 c.c., the final total volume in each tube being 2 c.c. Tubes were incubated two hours and placed in the refrigerator over night, readings being made next day. Culture controls as usual.

1. *Native agglutinins in normal rabbit serum.*—As is generally recognized agglutinins for various members of the typhoid-colon group of bacilli may be demonstrated in normal rabbit serum in low dilutions. As a control on our immune sera, we tested normal rabbit serum with all cultures using dilutions from 1:100, to 1:2,000. As expected, these dilutions were too high for the normal agglutinins of this group except for the following three cultures:

B. paratyphosus A. 1:400
B. paratyphosus A. 1:200
B. coli, Strain A. 1:100

2. *Titration of immune agglutinins.*—Each serum was then titrated with its own organism for ascertaining the exact agglutinin content. The results are given in Table 4.

3. *Cross-agglutination experiments.*—(a) For the purpose of comparing the specificity of the agglutinins and amboceptors and determining if any relation existed between the two in differentiating among members of the group, the immune sera of four cultures from the three subgroups were titrated against all cultures

of the general group. In Table 5 are given the results of such titration using the immune serum of *B. coli communior* with all cultures of the group.

TABLE 4.
TITRATION OF IMMUNE AGGLUTININS.

No.	Organism	1:100	1:200	1:400	1:800	1:1000	1:1500	1:2000	1:3000	1:4000	1:8000	1:16000	1:32000	1:64000
1	<i>B. coli communior</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
2	<i>B. coli communis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
3	<i>B. (lactis) aerogenes</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
4	<i>B. enteritidis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
5	<i>B. paracoli</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
6	<i>B. hog cholera</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
7	<i>B. alcaligenes</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
8	<i>B. paratyphosus A</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
9	<i>B. paratyphosus A</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
10	<i>B. paratyphosus B</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
11	<i>B. typhosus, Strain A</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
12	<i>B. typhosus, Strain B</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
13	<i>B. cholera</i>	+	+	+	+	+	+	+	+	+	+	+	+	+

TABLE 5.
CROSS-AGGLUTINATION WITH IMMUNE SERUM OF *B. coli communior*.

No.	Organism	1:100	1:200	1:400	1:600	1:800	1:1000	1:1500	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
1	<i>B. coli communior</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
2	<i>B. coli communis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
3	<i>B. (lactis) aerogenes</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
4	<i>B. enteritidis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
5	<i>B. paracoli</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
6	<i>B. hog cholera</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
7	<i>B. alcaligenes</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
8	<i>B. paratyphosus A</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
9	<i>B. paratyphosus A</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
10	<i>B. paratyphosus B</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
11	<i>B. typhosus, Strain A</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
12	<i>B. typhosus, Strain B</i>	+	+	+	+	+	+	+	+	+	+	+	+	+
13	<i>B. cholera</i>	+	+	+	+	+	+	+	+	+	+	+	+	+

It will be noted that the agglutinin for the particular strain of *B. coli* was quite specific for that strain. Also that this agglutinin gave best reactions with the members of the coli group and was generally negative for the remaining members of the general group except *B. paracoli* and *B. typhosus*, Strain A.

(b) The immune sera of two members of the "enteritidis group" (*B. enteritidis* and *B. paratyphosus A*) were titrated with all cultures with the results given in Tables 6 and 7:

It will be noted that in a general way these immune sera gave reactions best with members of their own (enteritidis) group and

then with the typhoid group, showing practically no agglutination of the coli group in the dilutions employed. The specific relationship between the agglutinin and its homologous culture (1:6400) is to be noted with *B. paratyphosus* A having agglutinated a closely allied culture, *B. paratyphosus* B, only in comparatively low dilution (1:1500).

TABLE 6.
CROSS-AGGLUTINATION WITH IMMUNE SERUM OF *B. enteritidis*.

No.	Organism	1:100	1:200	1:400	1:600	1:800	1:1000	1:2000	1:4000	1:8000
1	<i>B. coli communior</i>	—	—	—	—	—	—	—	—	—
2	<i>B. coli communis</i>	+	—	—	—	—	—	—	—	—
3	<i>B. aerogenes</i>	+	—	—	—	—	—	—	—	—
4	<i>B. enteritidis</i>	+	+	+	+	+	+	+	+	+
5	<i>B. paracoli</i>	+	—	—	—	—	—	—	—	—
6	<i>B. hog cholera</i>	+	±	—	—	—	—	—	—	—
7	<i>B. alcaligenes</i>	+	—	—	—	—	—	—	—	—
8	<i>B. paratyphosus</i> A.....	+	+	+	±	—	—	—	—	—
10	<i>B. paratyphosus</i> B.....	+	±	—	—	—	—	—	—	—
12	<i>B. typhosus</i>	+	±	—	—	—	—	—	—	—
13	<i>B. typhosus</i>	+	+	+	—	—	—	—	—	—
14	<i>B. cholera</i>	—	—	—	—	—	—	—	—	—

TABLE 7.
CROSS-AGGLUTINATION WITH IMMUNE SERUM OF *B. paratyphosus* A.

No.	Organism	1:100	1:200	1:400	1:600	1:800	1:1000	1:1500	1:2000	1:3000
1	<i>B. coli communior</i>	—	—	—	—	—	—	—	—	—
2	<i>B. coli communis</i>	—	—	—	—	—	—	—	—	—
3	<i>B. aerogenes</i>	—	—	—	—	—	—	—	—	—
4	<i>B. enteritidis</i>	+	+	±	—	—	—	—	—	—
5	<i>B. paracoli</i>	+	+	—	—	—	—	—	—	—
6	<i>B. hog cholera</i>	—	—	—	—	—	—	—	—	—
7	<i>B. alcaligenes</i>	—	—	—	—	—	—	—	—	—
8	<i>B. paratyphosus</i> A.....	+	+	+	+	+	+	+	+	+
10	<i>B. paratyphosus</i> B.....	+	+	±	+	+	+	±	—	—
12	<i>B. typhosus</i>	+	+	±	—	—	—	—	—	—
13	<i>B. typhosus</i>	+	+	—	—	—	—	—	—	—
14	<i>B. cholera</i>	—	—	—	—	—	—	—	—	—

(c) The immune serum of one member of the "typhoid group" (*B. typhosus*, Strain A) was titrated with all cultures as given in Table 8.

It will be noted in general that the agglutinin of this serum was no more potent for its own culture than for another strain of *B. typhosus*, that it agglutinated to some degree all members of the "enteritidis group" but caused no agglutination whatsoever of members of the "coli group."

(d) *Summary of agglutination experiments.*—(1) No native agglutinins for members of the typhoid-colon group of bacilli were

found in normal rabbit serum in dilution of 1:100 and higher except for *B. paratyphosus* A and *B. coli communior*

(2) Selecting the immune serum of *B. coli communior* as a representative of the "colon group" and titrating it with all cultures used in the study it was found that agglutination was best marked with the homologous culture. This serum also agglutinated the other members of the group, *B. coli communis* and *B. lactis aërogenes* in lower dilutions; it did not, however, agglutinate the members of the "enteritidis" and typhoid group except in two instances, *B. paracoli* (1:800) and *B. typhosus*, Strain A (1:100).

TABLE 8.
CROSS-AGGLUTINATION WITH IMMUNE SERUM OF *B. typhosus*, STRAIN A.

No.	Organism	1:100	1:200	1:400	1:600	1:800	1:1000	1:1500	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
1	<i>B. coli communior</i>	—	—	—	—	—	—	—	—	—	—	—	—	—
2	<i>B. coli communis</i>	—	—	—	—	—	—	—	—	—	—	—	—	—
3	<i>B. (lactis) aërogenes</i>	—	—	—	—	—	—	—	—	—	—	—	—	—
4	<i>B. enteritidis</i>	+	+	+	+	+	—	—	—	—	—	—	—	—
5	<i>B. paracoli</i>	+	+	+	+	+	—	—	—	—	—	—	—	—
6	<i>B. hog cholera</i>	+	+	±	—	—	—	—	—	—	—	—	—	—
7	<i>B. alcaligenes</i>	+	+	+	+	±	—	—	—	—	—	—	—	—
8	<i>B. paratyphosus</i> A	+	+	+	+	+	+	±	±	—	—	—	—	—
9	<i>B. paratyphosus</i> A	+	+	+	+	+	+	+	+	±	—	—	—	—
10	<i>B. paratyphosus</i> B	+	+	+	+	+	±	—	—	—	—	—	—	—
12	<i>B. typhosus</i> , Strain A	+	+	+	+	+	+	+	+	+	+	+	±	—
13	<i>B. typhosus</i> , Strain B	+	+	+	+	+	+	+	+	+	+	+	±	—
14	<i>B. cholera</i>	—	—	—	—	—	—	—	—	—	—	—	—	—

(3) Using the immune sera of *B. enteritidis* and *B. paratyphosus* A as representatives of the "enteritidis group" it will be noted in general that the cross-agglutination tests demonstrated the close relationship of this group to the "typhoid" rather than to the "coli" group. There is apparently a closer interrelationship among *B. enteritidis*, *B. paracoli*, *B. paratyphosus* A and B, and *B. typhosus* than with any other members of the typhoid-colon group.

(4) Titrating the immune serum of a strain of *B. typhosus* with all cultures it was found that agglutination with the homologous culture did not exceed that resulting with another strain of *B. typhosus* and demonstrated by means of agglutination of members of the "enteritidis" group the close interrelationship of the "typhoid" with the "enteritidis group." The negative results

with the "coli" group would indicate that the colon and typhoid bacilli are entirely separate entities or that the relationship is very distant. These results are substantiated by the complement-fixation reactions.

COMPLEMENT-FIXATION EXPERIMENTS.

Of serological reactions, that of complement-fixation should be most accurate and specific as indicating the presence of immune amboceptor, the result of cellular changes incident to the introduction of bacterial or any other protein antigenic substances. As is well known, the agglutinins produced by members of the typhoid-colon group are more or less specific in the higher dilutions, yielding in most instances a group reaction in lower dilutions. The object of these experiments was to test the specificity of the amboceptors as compared to the agglutinins and to indicate in this manner the biological relationship of the members to each other.

Method.—Briefly this consisted in testing each culture antigen with all immune sera. The best reactions would likely occur between homologous antigen and immune serum, and the subsidiary reactions occur if amboceptor for the antigen in question were present in the other immune sera. To study the specificity of the amboceptors two methods were employed: (a) using a constant dose of antigen and increasing doses of immune sera and (b) using a constant amount of immune serum with increasing or decreasing doses of antigen.

(1) *Antigens.*—The preparation of the antigens in experiments of this nature is an important feature. The specific antigenic principle may be in the nature of an endotoxin and apart from the general protein structure of the bacterial cell. The method of Besredka as modified by Gay has this possibility in view in the preparation of bacterial antigens. On the other hand the organism may secrete a substance diffusible in the culture media which may represent the more specific antigenic principle. Both methods were used in this study, the latter being chosen for most of the work because much more readily prepared and yielding apparently the same results as secured by the Besredka method.

Method No. 1.—Cultures were grown in neutral broth for 48

hours and filtered to remove clumps of organisms. The filtrate was then heated to 60° C. for an hour. One per cent phenol and glycerin were added as preservatives. Antigens were then titrated for antigenic and anticomplementary doses. These antigens are very simple in preparation and keep for long periods of time in the ice-chest.

Method No. 2 (Besredka-Gay).—Cultures were grown on slanted neutral agar for 48 hours. The growths were washed off with small amounts of sterile salt solution. The saline suspensions were precipitated with an equal quantity of absolute alcohol and centrifugalized. The sediment was then dried over calcium chlorid, accurately weighed, ground into a fine powder and made up into a 2 per cent suspension in 0.85 per cent salt solution. This suspension was diluted with sterile salt solution to 1/40, 1/80, 1/160, 1/320, 1/640, and 1/1280 and 1 c.c. of these dilutions used in each tube of the experiment as shown in the tables. The actual amounts of dry antigenic substance contained in 1 c.c. as used were as follows:

1/40	dilution = 0.5	mg.
1/80	" = 0.25	mg.
1/160	" = 0.125	mg.
1/320	" = 0.062	mg.
1/640	" = 0.031	mg.
1/1280	" = 0.0155	mg.

In using an antigen so prepared each dose was controlled to guard against anticomplementary effects. Several antigens were used in larger doses but so many were anticomplementary that we were forced to use the higher dilutions given above.

With antigens made according to the first technic two methods may be used in conducting complement-fixation reactions: (a) Ascertaining the anticomplementary dose and adopting an arbitrary amount which is less than the anticomplementary dose or (b) titrating the exact antigenic dose of the antigen with its immune serum. This dose is selected as that giving complete inhibition of hemolysis and must be several times less than the anticomplementary dose. As will be seen in the accompanying table of these titrations, this method of preparing antigens yielded good results, i.e., the anticomplementary was from 10 to 20 times greater than the antigenic

TABLE 6.
DETERMINATION OF ANTICOMPLEMENTARY AND FIXING POWER OF THE DIFFERENT ANTIGENS.
ANTICOMPLEMENTARY POWER.

DOSE OF SERUM	AMOUNT OF ANTIGEN c.c.	ANTIGENS													
		1 1:10	2 1:10	3	4	5	6 1:10	7 1:10	8	10	11 1:10	12	13	14	
	0.1	—	—	—	—	—	—	—	—	—	—	—	—	—	
	0.2	—	—	—	—	—	—	—	—	—	—	—	—	—	
	0.4	—	—	—	—	—	—	—	—	—	—	—	—	—	
	0.6	—	—	—	—	—	—	—	—	—	—	—	—	—	
	0.8	—	—	—	—	—	—	—	—	—	—	—	—	—	
	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—	
	2.0	—	—	—	—	—	—	—	—	—	—	—	—	—	
	3.0	—	—	—	—	—	—	—	—	—	—	—	—	—	

FIXING POWER.

IMMUNE SERUM c.c.	Antigenic dose used (undiluted)														
		1 0.1 c.c.	2 0.2 c.c.	3 0.3 c.c.	4 0.4 c.c.	5 0.5 c.c.	6 0.6 c.c.	7 0.7 c.c.	8 0.8 c.c.	9 0.9 c.c.	10 1.0 c.c.	11 1.1 c.c.	12 1.2 c.c.	13 1.3 c.c.	14 1.4 c.c.
0.005	0.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"	0.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"	0.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"	0.6	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"	0.8	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"	2.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Complete hemolysis. S.H. : Slight inhibition of hemolysis. I.H. : Complete inhibition of hemolysis. M.H. : Marked inhibition of hemolysis.

dose. It appeared to us that since we were searching for the most delicate reactions between an antigen and any possible amboceptor for this antigen which may be present in other immune sera, it would be better technic to use the antigenic dose and vary the quantity of immune serum.

(2) *Immune sera*.—These were inactivated by heating to 55° C. for half an hour. In titrating the antigenic doses of the various antigens, 0.005 c.c. of the homologous immune serum was used in each tube (Table 8). In conducting the reactions the titrated antigenic dose of the antigen being tested was employed with increasing doses of the immune sera, as follows: 0.001 c.c., 0.004 c.c., 0.008 c.c., 0.01 c.c., and 0.02 c.c.

(3) *Hemolytic system*.—Complement was furnished by the fresh sera of guinea-pigs, diluted 1:20 and used in dose of 1 c.c.; antishoop rabbit amboceptor was titrated before each experiment and used in amounts equal to two hemolytic doses; washed sheep corpuscles were employed in a 2.5 per cent suspension, dose 1 c.c.

Serum, antigen, and complement were mixed and diluted with NaCl solution to 3 c.c.; incubated one hour at 37° C.; two hemolytic doses of amboceptor and 1 c.c. corpuscles added; reincubated for one to two hours, depending upon the hemolysis of the controls; tubes were then placed in refrigerator over night, readings being made the following morning.

(4) *Controls*.—Controls of each serum in maximum dose used also antigen, complement, corpuscles, and hemolytic controls. A normal serum was used each time with each antigen. In the doses of sera used by us we did not note the presence of native amboceptor for any of the antigens except in one instance: *B. paracoli* which yielded a weak reaction with the maximum dose of serum (Table 14).

The following tables show the results. As already stated the numbers are the same for each immune serum and its antigen, thus, No. 1 refers to culture No. 1 (*B. coli communior*), and its immune serum (No. 1), etc.

A study of Tables 10-22 shows the following:

1. The immune amboceptor for *B. coli* is highly specific with its own antigen. While the two strains used were slightly different in their fermentative powers with the various sugars, they may be

TABLE 10.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN NO. 1.
(*B. coli communior*.)

[illegible]

TABLE II.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN NO. 2.
(*B. coli communis*.)

[illegible]

TABLE 12.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN NO. 3.
(*B. lactis aërogenes*.)

DOSE IMMUNE SERUM C.C.	ANTIGEN No. 3 C.C.	IMMUNE SERA												NORMAL SERUM
		I	2	3	4	5	6	7	8	10	11	12	13	
0.001.....	0.1	—	—	M.I.H.	—	—	—	—	—	—	—	—	—	
0.004.....	"	—	—	I.H.	—	—	—	—	—	—	—	—	—	
0.008.....	"	—	—	I.H.	—	—	—	—	—	—	—	—	—	
0.01.....	"	—	—	I.H.	S.I.H. M.I.H.	—	—	—	—	—	—	—	—	
0.02.....	"	—	—	I.H.	—	—	—	—	—	—	—	—	—	
0.02.....	None	—	—	—	—	S.I.H.	—	—	—	—	—	—	—	

TABLE 13.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN No. 4.
(*B. enteridis*.)

[illegible]

TABLE 14.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN NO. 5.
(*B. paracoli*.)

[illegible]

TABLE 15.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN No. 6.
(*B. hog cholera*.)

DOSE IMMUNE SERUM C.C.	ANTIGEN No. 6 C.C.	IMMUNE SERA											NORMAL SERUM
		1	2	3	4	5	6	7	8	10	11	12	
0.001	0.08	—	—	—	—	—	S.I.H.	—	—	—	—	—	—
0.004	"	—	—	—	—	—	S.I.H.	—	—	—	—	—	—
0.008	"	—	—	—	—	—	M.I.H.	—	—	—	—	—	—
0.01	"	—	—	—	—	—	M.I.H.	—	—	—	—	—	—
0.02	"	—	—	—	—	—	I.I.L.	—	—	—	—	—	—
0.02	None	—	—	—	—	—	—	—	—	—	—	—	—

TABLE 16.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN No. 7.
(*B. fecalis alcaligenes*.)

[illegible]

TABLE 17.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN NO. 8.
(*B. paratyphosus* A.)

[illegible]

TABLE 18.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN NO. 10.
(*B. paratyphosus* B.)

DOSE IMMUNE SERUM C.C.	ANTIGEN No. II C.C.	IMMUNE SERA											NORMAL SERUM
		1	2	3	4	5	6	7	8	10	11	12	
0.001.....	.025	—	—	—	—	—	—	—	—	—	—	—	—
0.004.....	"	—	—	—	—	—	—	—	—	S.I.H.	—	—	—
0.008.....	"	—	—	—	—	—	—	—	—	I.H.	—	—	—
0.01.....	"	—	—	—	—	—	—	—	—	I.H.	—	—	—
0.02.....	"	—	—	—	—	—	—	—	—	I.H.	—	—	—
0.02.....	None	—	—	—	S.I.H.	S.I.H.	—	—	S.I.H.	—	V.S.I.H.	—	—

TABLE 19.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN NO. 11.
(*B. dysenterica* Flexner.)

[illegible]

TABLE 20.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN No. 12.
(*B. typhosus* A.)

[illegible]

TABLE 21.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN No. 13.
(*B. typhosus*, Strain B.)

[illegible]

regarded as closely allied organisms, altho it will be noted in Tables 10 and 11 that their amboceptors reacted only with the homologous antigen. The specificity of the agglutinins is not as marked as with the amboceptors because some agglutination occurred with other members of the group in crossing with the immune serum of *B. coli communior*: for example, *B. coli communis*, *B. aërogenes*, and *B. paracoli*. It is to be noted throughout the study that the coli immune sera did not in general contain amboceptor for other members of the group.

2. The antigen of *B. lactis aërogenes* reacted best with its homologous immune serum and to a lesser extent with the immune sera of *B. enteritidis* and *B. hog cholera*. While this bacillus is classed with the coli group the complement-fixation reactions would indicate a clear relationship to the above organisms.

3. The antigen of *B. enteritidis* reacted best with its own immune serum and that of *B. paracoli* and to a lesser extent with the sera of *B. typhosus*, *B. paratyphosus* A, and *B. aërogenes*. The immune serum of this bacillus likewise agglutinated the above cultures but there is apparently no relation between the quantities of agglutinins and amboceptors present. The apparent close interrelationship of *B. enteritidis* to *B. paracoli*, *B. aërogenes*, and the paratyphoids will be noted throughout the experiments.

4. The close interrelationship of *B. paracoli* to *B. enteritidis*, *B. typhosus*, and *B. aërogenes* is indicated by the amboceptors for this organism present in the immune sera of these cultures.

5. The antigens of *B. hog cholera* and *B. alcaligenes* reacted only with their immune sera and the absence of amboceptors for these antigens in the immune sera of the other members of the group may be regarded as indicating, serologically at least, their more distant relationship to the members of the colon-typhoid group.

6. The higher specificity of immune amboceptor over agglutinin is apparent by comparing Tables 7 and 17, when it will be noted that the antigen of *B. paratyphosus* A reacted strongly with its own immune serum and to a slight degree with *B. paratyphosus* B, *B. typhosus*, *B. enteritidis*, etc. The immune serum of this antigen agglutinated practically the same organisms but the differentiation was less sharply defined. The more specific character of the ambo-

ceptor is also noted by the well marked reaction between the antigen of *B. paratyphosus* B, and its immune serum, and very slight reactions with the sera of *B. paratyphosus* A, *B. typhosus*, *B. enteritidis*, and *B. paracoli*.

7. The antigen of *B. typhosus* (Strain A) reacted as well with the immune serum of Strain B as with its homologous serum. Reactions also occurred with the immune sera of the same cultures as were agglutinated by the serum of Strain A, altho there is apparently no quantitative relationship. The antigen of Strain B reacted more specifically, giving strong complement-fixation with the amboceptor of its own serum and that of Strain A, and yielding slighter reactions with the immune serum of *B. paracoli* and *B. enteritidis*.

8. Since several varieties of *B. dysenterica* are usually classified with the typhoid group it was considered of interest to prepare an antigen of at least one well known strain and test for amboceptor in the immune sera of the series. As will be noted in Table 19 best reactions occurred with *B. enteritidis*, *B. paracoli*, and *B. aërogenes*, very slight reactions resulting with immune sera of *B. typhosus* and the paratyphoids. This would indicate, serologically, the closer relationship of this strain to *B. enteritidis* and *B. paracoli*.

9. As a control on the investigation a culture of cholera was carried along with the series. This organism was selected because it is an inhabitant of the intestinal tract as are the members of the typhoid-colon group altho readily differentiated from these by biological characters and agglutination tests. It will be noted that the cholera immune serum did not show the presence of any amboceptor for the members of the typhoid-colon group altho its antigen yielded very slight reactions with the immune sera of *B. aërogenes*, *B. enteritidis*, and *B. paracoli*.

There was practically no difference in results with the use of antigens prepared according to the method of Besredka as modified by Gay. This method is probably the best we have for the purpose of liberating any specific antigenic substance as an endotoxin which would serve to yield more specific reaction and differentiate among the members of a group of closely allied organisms. The three specimen tables, 23, 24, and 25, compared in order with Tables 10, 18, and 20, show that results were practically the same.

TABLE 23.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN No. 1.
(*B. coli communior*) (Besredka Method).

Amount of Antigen Gm.	Amount of Im- mune Serum c.c.	1	3	4	5	6	7	10	12	13	Normal serum
0.0155.....	0.1	—	—	—	—	—	—	—	—	—	—
0.031.....	"	—	—	—	—	—	—	—	—	—	—
0.062.....	"	—	—	—	—	—	—	—	—	—	—
0.125.....	"	—	—	—	—	—	—	—	—	—	—
0.25.....	"	M.I.H.	—	—	—	—	—	S.I.H.	—	—	—
0.5.....	"	I.H.	—	—	—	—	—	—	—	—	—
None.....	—	—	—	—	—	—	—	—	—	—

TABLE 24.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN No. 10.
(*B. paratyphosus B*) (Besredka Method).

Amount of Antigen Gm.	Amount of Im- mune Serum c.c.	1	3	4	5	6	7	11	12	13	Normal Serum
0.0155.....	0.1	—	—	—	—	—	—	—	—	—	—
0.031.....	"	—	—	—	—	—	—	—	—	—	—
0.062.....	"	—	—	—	—	—	—	—	—	—	—
0.125.....	"	—	—	—	—	—	—	—	—	—	—
0.25.....	"	—	—	S.I.H.	—	—	—	S.I.H.	—	—	—
0.5.....	"	—	S.I.H.	S.I.H.	S.I.H.	—	—	M.I.H.	S.I.H.	—	—
None.....	—	—	—	—	—	—	I.H.	—	—	—

TABLE 25.
FIXING POWER OF IMMUNE SERA WITH ANTIGEN No. 12.
(*B. typhosus*, Strain A) (Bestredka Method).

[illegible]

Having the full set of antigens on hand it was considered of interest to try out a number of sera of typhoid-fever patients to ascertain the specificity of complement-fixation experiments as a practical diagnostic procedure in the presence of infection with a member of the typhoid-colon group. In this experiment the antigens were used in one-quarter the anticomplementary dose as representing the simplest practical method. The sera were titrated with a culture of *B. typhosus* (Strain B) for agglutinin content; were then inactivated and used in dose of 0.1 c.c. for the complement-fixation experiment. As usual, serum, antigen, hemolytic, etc., controls were set up. The results are given in Table 26.

It will be noted (1) that with each serum the strongest reactions occurred with the culture antigens of the two strains of *B. typhosus*; (2) that according to these results, complement-fixation tests with these antigens are essentially group reactions; (3) that the same interrelationship among *B. typhosus*, *B. paratyphosus*, *B. enteritidis*, and *B. paracoli*, as was noted with the immune rabbit sera, is here shown.

GENERAL CONCLUSIONS.

1. The absolute differentiation among members of a "group" of organisms by complement-fixation reactions does not seem possible. The organisms belonging to such "groups" possess antigenic principles in common which produce a more general amboceptor and yield essentially a "group" reaction. In differentiating among various "groups," however, complement-fixation reactions are probably the most exact at our command. The work of Craig and Nickols with several cultures of spirochetes including *Treponema pallidum*, of Claypole with different strains of streptothrices, and the work with the group of streptococci, diphtheria, and typhoid-colon group, all indicate that complement-fixation reactions with bacterial antigens and immune amboceptors are essentially "group" reactions.

2. As between agglutination and complement-fixation reactions in differentiating among the individual species of a certain group of organisms, results would indicate that the amboceptors are more

TABLE 26.
FIXING POWER OF SERA OF TYPHOID-FEVER PATIENTS WITH THE VARIOUS ANTIGENS.

No.	STAGE OF DISEASE	DOSE OF SERUM C.C.	ANTIGENS													TITRE OF AGGLUTININ
			ANTIGENIC DOSE: 1 THE ANTICOMPLEMENTARY													
			1	2	3	4	5	7	10	11	12	13				
1.....	Convalescent	0.1	S.H.	S.H.	S.H.	M.H.	M.H.	M.H.	S.H.	M.H.	I.H.	I.H.	1:2500			
2.....	"	"	—	S.H.	V.S.H.	I.H.	I.H.	S.H.	V.S.H.	S.H.	M.H.	I.H.	1:2500			
3.....	"	"	V.S.H.	V.S.H.	S.H.	I.H.	I.H.	S.H.	V.S.H.	M.H.	I.H.	I.H.	1:1000			
4.....	"	"	S.H.	S.H.	S.H.	M.H.	M.H.	S.H.	S.H.	S.H.	I.H.	I.H.	1:4000			
5.....	"	"	I.H.	○	○	○	○	○	○	○	I.H.	I.H.	○			

specific than the agglutinins, or in other words, that complement-fixation reactions with the proper technic are more delicate than agglutination reactions. As a practical procedure, however, the agglutination reactions are so much simpler that the more complicated and time-consuming fixation technic is not serviceable as a practical routine procedure.

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ON THE VALUE OF THE "ABORTIN" AS A DIAGNOSTIC AGENT FOR INFECTIOUS ABORTION IN CATTLE.*

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During his experiments on infectious abortion, Bang¹ observed that artificially infected animals, following a subcutaneous injection of a serum broth culture, reacted with a marked febrile rise of temperature, associated with inappetency and slight diarrhea. Based on another experiment, conducted on a healthy bull calf, which also reacted to an injection of the same material, the investigator concluded that the febrile reaction has no specific diagnostic value. Unfortunately Bang has not yet reported the experiments he intended to conduct with sterile cultures.

Recently the English Commission, M'Fadyean and Stockman,² mentioned, in their report on epizootic abortion, noteworthy results with the glycerin extract of *B. abortus*, it being their intention to prepare a diagnostic agent similar to those known as tuberculin and

* Received for publication July 7, 1913.

¹ *Ztschr. f. Tiermed.*, 1897, 1, p. 241; *Archiv. f. Wissenschaft und Prakt. Tierheilk.*, 1907, 33, p. 312.

² *Report of Departmental Committee on Epizootic Abortion*, Part I, "Epizootic Abortion in Cattle," and Appendix to Part I, "Epizootic Abortion in Cattle," London, 1909; *Jour. Comp. Path. and Therap.*, 1910, 23, p. 370.

mallein. The biological product they obtained was called by them "abortin." The technic of its preparation was given as follows:

A 1 per cent glucose serum glycerin broth, infected with *B. abortus*, was grown for 6 weeks at 37° C., sterilized for 2 hours at 99° C., then filtered through filter paper or Berkefeld candles, and 1 per cent carbolic acid added, or, if not used immediately, concentrated on the water bath to one-tenth of its volume.

This preparation, injected subcutaneously or intravenously, produced in infected animals a rise of temperature which began about the fourth hour and lasted about 14 hours. The intravenous injection was frequently accompanied by rather alarming general symptoms, similar to those of an anaphylactic shock. The subcutaneous injection with 0.5 to 1 c.c. "abortin brüte" was, however, harmless. In normal animals no reactions were recorded. Animals which had aborted as a sequel to the infection with *B. abortus* still reacted several months afterward. Basing their conclusions on their results, the English Commission considers the "abortin reaction" to be a specific one. This publication has been the subject of considerable comment, and in various laboratories an attempt was made to confirm or to deny the diagnostic value of "abortin."

Belfanti,¹ in comparing the value of a few diagnostic agents for infectious abortion, came to the conclusion that the "abortin" should be considered as an unreliable diagnostic agent until contrary proofs are given. He doubts the specificity of the reaction. From his publication the following results are interesting:

Out of 19 animals tested, 10 reacted to the "abortin"; only 7 were positive to the serum test; 6 did not react to the "abortin," yet only 3 were negative to the serum test. Three doubtful "abortin" reactions belong to the group of 9 animals which were doubtful to the serum tests.

The preparation he used came from the laboratories of Jensen and Zwick, and his own make. Zwick and Zeller,² in their experiments with "abortin," reached the conclusion that the "abortin" is not suited as a diagnostic for the determination of infectious abortion. Frequently the "abortin" reactions did not correspond with the serum reactions. Their publication is largely responsible for our own work, and there will be later opportunities offered to

¹ *Ztschr. f. Infektionskr., d. Haustiere*, 1912, 12, p. 1.

² *Arb. a. d. k. Gsndtsamte.*, 1912, 43, p. 95.

discuss some of their results in connection with ours. Holth¹ expresses the opinion that possibly the "abortin" will be a useful, perhaps more delicate, reagent for the determination of early stages of the abortus infection than the usual, reliable immunity reactions. Caspar,² Brüll,³ DeVine,⁴ and others report, without going into detailed account of their work, only unreliable results. Mohler and Traum⁵ in this country report failures. Giltner,⁶ in his work on "abortin," has, unfortunately, carried out no serum tests, and, therefore, his results are not suitable for comparison.

In general these results were not as claimed by M'Fadyean and Stockman, and, as it was our intention to form our own independent idea as to the action and importance of the "abortin" as a diagnostic agent, a series of experiments was undertaken. This work was readily possible, as the laboratory for the last two and one-half years has been engaged in a detailed study of infectious abortion of cattle. Furthermore, as inquiries were constantly received regarding the value of the "abortin" which is at present commercially distributed in this country, a most thorough investigation was urgently demanded.

THE PREPARATION OF "ABORTIN."

Different culture strains, isolated by ourselves from fetuses, and fetal membranes, grown on glycerin-bile-serum agar, were used. Some of these strains were extremely virulent to rats, where others proved to be extremely low in virulence, and consequently also of low toxicity. This fact was determined only after some "abortin" prepared with such strains had been used for tests. The general technic in preparing the "abortin" was as follows:

So-called Holth flasks,⁷ containing glycerin broth of the same reaction (+1.2) as used for the preparation of tuberculin in the laboratory, were inoculated with *B. abortus*. The flask, properly saturated with oxygen, as explained by Holth, was kept

¹ *Ztschr. f. Infektionskr., d. Haustiere*, 1911, 10, p. 342.

² *Deutsche tierärztl. Wchnschr.*, 1911, 19, p. 785.

³ *Berliner tierärztl. Wchnschr.*, 1911, 27, p. 721.

⁴ *Veterinary News*, 1910, 7, p. 697.

⁵ *Annual Report, Bureau of Animal Industry*, 1911, p. 175.

⁶ *Proceedings of the A.V.M.A.*, 1912, p. 345.

⁷ *Ztschr. f. Infektionskr., d. Haustiere*, 1911, 10, p. 217.

at 37° C. for from 6 to 16 weeks. An addition of grape sugar or serum did not improve the growth or the quality of the preparation. Liver broth gave uniformly better results than beef broth. After the time mentioned above had elapsed, and an extremely heavy flocculent deposit was found in the flasks, the cultures were tested for purity. The supernatant fluid was usually turbid at the beginning of the growth, but cleared up gradually as the culture became older. The pure cultures of different strains were poured together, killed by keeping them for one hour in the board of health sterilizer, and then concentrated if possible by vacuum at 60° to 70° C. to one-tenth of its volume. The slow extraction at a low temperature seems also for "abortin" to be essential for a good preparation. The syrupy fluid was filtered through hardened filter paper, and kept in sterile brown bottles at a temperature of 8° C. For use it was always diluted with a saline solution containing 1 per cent glycerin and 0.5 per cent carbolic acid.

As will be shown by the detailed account of our experiments, we found that a "purified abortin" acts more specifically than the preparations just mentioned. From our observations in the conjunctival test for glanders, we realized that an old preparation, particularly when kept in powder form, is more specific. This is undoubtedly due to the fact that the unspecific pyrogenous substances disintegrate rather quickly. A special diagnostic, the so-called "precipitated dry abortin," was prepared. Its manufacture was in our hands as follows:

Twelve-weeks-old cultures, obtained as above, and concentrated at 40° C. to one-tenth of its volume, were first centrifuged and then filtered. A comparatively clear brownish syrupy fluid was obtained. One part of this liquid was precipitated with 20 parts of absolute alcohol, and washed with absolute alcohol and ether. The precipitate, filtered off, was smeared on sterile clay plates and dried over sulfuric acid in vacuum. A granular whitish powder was finally obtained.

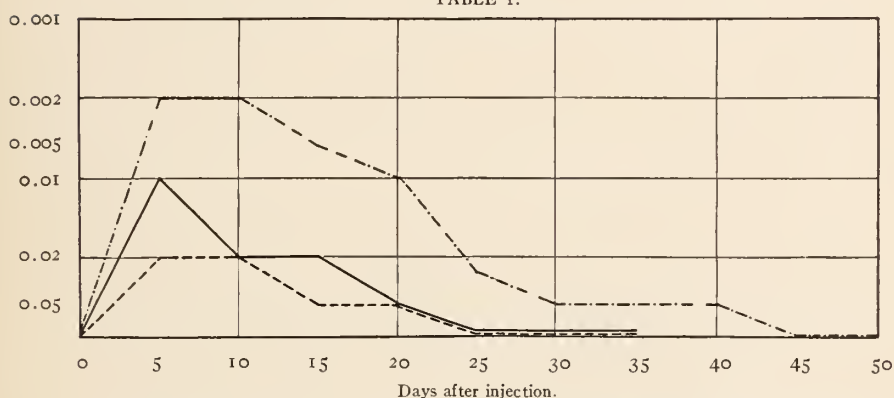
This preparation is not hygroscopic, and is very readily soluble in saline solution. Out of 10 c.c. "abortin brute," that is, the above-named concentrated glycerin broth culture, about 0.95 gm. of powder was received. This preparation kept well in brown bottles, in an exiccator. For the experiments it was dissolved in saline solution.

To test the antigenic properties of our various preparations 3 rabbits for each preparation were inoculated intravenously with 1 c.c. "abortin brute" (10 c.c. diluted or 0.1 gram precipitated "abortin" in 1 c.c. saline). The results are shown graphically in Table 1.

These comparatively few tests show that the highest amount of antigen is found in the "precipitated abortin." The immune

bodies appeared in the rabbits, between the second and the fifth day, remained present for about 10-15 days, but after 45 days had entirely disappeared. Ordinary "abortin brute" from rather young cultures produced only a very small amount of immune bodies; they disappeared in 25 days from the blood of the inoculated animals. "Abortin" from old cultures produced more immune bodies, but these disappeared as quickly as the others. These observations stand in correlation with similar experiments of Zwick and Zeller.

TABLE 1.



Graphic demonstration of the average agglutination titre of 12 rabbits which have been inoculated with the "abortin" preparation used for the tests. One c.c. "abortin brute" (=10 c.c. diluted or filtrate, or 0.1 gram precipitate in 1 c.c. saline) was injected intravenously.

— = W/4 strain 47-days-old culture.

----- = 2445/3 strain 4-weeks growth.

-.-.-.- = Na 2916 strain 92-days-old culture, precipitated and kept in powder form.

From these preliminary tests we conclude that the "precipitated dry abortin," particularly from old cultures, has greater antigenic properties than the ordinary "abortin brute," a point which is noteworthy when comparing later the practical test results obtained with these products.

In addition to these tests on rabbits, the "abortin" was also used as an antigen in the complement fixation test, with a standard serum of the titre 0.002. Uniformly the antigen titrated to 0.01 c.c., and the "abortin" had a titre of 0.01. In this test we found that no difference exists between the titre of the "ordinary abortin brute" and the "precipitated abortin." The technic of this test,

which undoubtedly permits a standardization of the "abortin" preparations, is extremely simple. Descending doses of "abortin" are tested with complement, hemolysin, and blood. At the same time the same amount of "abortin" is brought in contact with three units of a standard serum, in our particular case 0.006 c.c. serum. Titrated amounts of complement, hemolysins, and blood are added. We can recommend this method based on a large number of tests.

THE TESTS WITH "ABORTIN."

In conjunction with the "abortin" test, naturally serum tests were carried out. The technic for the latter is the same as recommended by Sven Wall¹ and Holth.² Since 1912 these serum tests for infectious abortion have been used in our laboratory as the practical routine tests. They have given uniformly accurate and valuable results.

Before the "abortin" was applied, in every instance blood was collected for the serum tests. For some of the tests we were unable, on account of the large amount of other routine work, to carry out both the agglutination and complement fixation tests simultaneously; as the complement fixation test is more reliable for immunity tests than the agglutination test, it was, therefore, applied as a single test. If in any way possible a retest was made 6 to 12 months after the first test. The interpretation of these tests was the same as recommended and proven by Sven Wall, namely, agglutination ≥ 0.05 and complement fixation ≥ 0.05 is considered a positive reaction. For the agglutination reaction a titre of ≥ 0.01 and for the complement fixation test a reaction with ≥ 0.05 was accepted as positive.

GENERAL TECHNIC OF THE "ABORTIN" TESTS.

In a similar manner as recommended for the tuberculin test two to three preliminary temperatures were taken. The injection of the "abortin" was made subcutaneously or intravenously. Following the injection the temperature readings were made at intervals of two hours. The last temperature was taken usually at the

¹ *Ztschr. f. Infektionskr., d. Haustiere*, 1911, 10, pp. 23 and 132.

Ibid., pp. 206 and 342.

eighteenth or twenty-fourth hour. If, following the "abortin" injection, a rise of temperature was recorded it began between the fourth and twelfth hour, and remained at its height for from 2-16 hours. Occasionally "late" and "atypical" reactions were also recorded. The interpretation of these reactions was made according to the rules adopted by the Eighth International Veterinary Congress in Budapest; namely, an increase of temperature above 40° C. indicates a reaction, if, at the time of the "abortin" injection, the temperature is not over 39.5° C. For the recording of the temperatures "Reform" thermometers (celsius) or "Fahrenheit" thermometers (often two or three at a time) were used.

Local reactions were never noticed as long as only quantities of 1 c.c. "abortin brute" were inoculated. In some instances 5 or 10 c.c. of this preparation were applied. A large, hard, and painful swelling developed which disappeared very slowly. Often 2 to 3 weeks elapsed before it was entirely absorbed. Never was any abscess formation noticed.

Some of the animals, following the intravenous injection, showed general symptoms, trembling, slobbering, accelerated respiration, inappetency, and a reduced secretion of milk. The number of these occurrences was very small. For further details we refer to the experiments.

At this time we have tested altogether 270 animals naturally infected with *B. abortus*. Also some artificially infected animals were tested. The results of these tests were as follows:

"ABORTIN" TEST ON ARTIFICIALLY INFECTED ANIMALS.

For the test 3 heifers, Nos. 45, 46, and 47, stood at disposal. The serum tests showed that the animals had a high agglutination and complement fixation titre. The application of the "abortin" was made subcutaneously. The result of this test is shown in Table 2.

In considering the rules mentioned, Heifers 45 and 46 gave a positive reaction to the subcutaneous "abortin" injection. Heifer 47, which was fed with vaginal discharge, failed to give a reaction. This fact is rather astonishing inasmuch as an agglutination reaction of 0.002 and a complement fixation of 0.02 were recorded.

These experiments show that the "abortin" is not an absolutely reliable diagnostic for artificially infected animals.

"ABORTIN" TEST ON NATURALLY INFECTED ANIMALS.

Herd No. 1.—The dairy herd of the Experimental Farm, numbering 49 animals, in which infectious abortion has existed for the past five years, was used for this test.

TABLE 2.

ANIMAL NUMBER	MODE OF INJECTION	SERUM TESTS AUG. 2, 1912		TEMPERATURES BEFORE INJECTION AUG. 2, 1912			TEMPERATURES AFTER SUBCUTANEOUS INJECTION OF 10 C.C. OF ABORTIN AT THE NECK AT 8:00 A.M.								RESULT OF ABORTIN TEST	HISTORY	
		Agglu- tination	Comple- ment Fixation	5:00 A.M.	6:00 A.M.	7:00 A.M.	10:00 A.M.	12:00 M.	2:00 P.M.	4:00 P.M.	6:00 P.M.	8:00 P.M.	10:00 P.M.	12:00 P.M.			2:00 A.M.
45.	100 c.c. broth cul- ture, 6/1/12, intra- venously.	> 0.001	= 0.05	100.2	100.6	100.4	102.3	101.6	102.4	103.2	103.6	104.4	104.4	102.4	102.4	Positive	Premature calf, 11/8/ 12. <i>B. abortus</i> isolated from fetal membranes.
46.	100 c.c. broth cul- ture, 6/1/12, intra- venously.	> 0.001	= 0.05	102.2	102.	101.8	101.8	103.1	104.	104.3	103.8	102.4	102.5	102.9	102.2	Positive	Calved normally, 12/ 31/12. <i>B. abortus</i> isolated from cotyle- dons.
47.	Fed vaginal discharge of several aborters for ten days; 5/1/12	= 0.002	= 0.02	101.3	101.2	101.4	101.2	101.4	101.5	102.2	102.8	102.	101.8	102.2	101.7	Was bred in May, 1913, for the first time with success. Aborted probably very small fetus.

The "abortin" applied was a mixture of 4 different cultures, was not concentrated, but simply passed through Berkefeld candles and preserved with 0.5 per cent carbolic acid. The injection was made subcutaneously. The results of the test are shown in Table 3.

At the time of the test 23 animals (Nos. 1, 2, 6, 7, 9, 10, 12, 14, 16, 17, 19, 20, 21, 29, 31, 34, 41, 42, 45, 46, 47, 48, 49) reacted to the serum test. Out of these reactors 10 had aborted (Nos. 6, 7, 9, 16, 17, 19, 45, 46, 48, 49). By means of the "abortin" test 13 animals (Nos. 4, 13, 14, 20, 25, 26, 27, 29, 31, 32, 33, 46, 48) were found to react to the inoculation, with fever over 104° F. and with a curve which is characteristic for a positive tuberculin reaction. The differences in temperature ranged from 1.6° to 5.2° F. Out of these 13 animals six (Nos. 14, 20, 29, 31, 46, 48) were reactors to the serum test, whereas seven (Nos. 4, 13, 25, 26, 27, 32, 33), which also reacted to the inoculation, were, according to the serum tests, not infected with *B. abortus*. Several animals (Nos. 16, 17, 45, and 49), which had aborted during the last year, and of which Nos. 16, 45, and 49 gave positive reactions to the serum tests, failed to react to the "abortin." No local reactions nor general symptoms were noticed.

Conclusions.—The test applied to Herd No. 1 showed that the inoculation of "abortin" is a very unreliable method of diagnosing the existence of the disease in a herd, particularly when broth filtrates are applied subcutaneously. The results show that a particularly high percentage (53.8 per cent) of the entire "abortin" reactions are found in the non-infected, healthy animals. The test, therefore, is considered extremely unreliable and unspecific with this preparation. An interesting feature has to be mentioned here, namely, several of the animals, which had reacted to the "abortin," but were not affected with *B. abortus*, according to the serum tests, had been subjects of various tests with tuberculin preparations, or had been vaccinated with large doses of human tubercle bacilli according to the method of Pearson. Several of the animals were also badly affected with tuberculosis as subsequent detailed autopsies revealed. We are not in a position to explain satisfactorily this occurrence of reaction in such animals, but suspect that they are probably due to proteins or split products present in the broth.

Herd No. 2.—A dairy herd, consisting of 19 animals, in which recently 2 cases (Nos. 6 and 8) of infectious abortion had been bacteriologically determined, was tested with "abortin." The preparation used was a mixture of cultures similar to that used in Herd No. 1, but concentrated to one-tenth of its volume, and the "abortin brüte" dissolved in the quantity of 1-9 c.c. dilution fluid, as mentioned in the introduction. Each 10 c.c. contained, therefore, 1 c.c. of "abortin brüte." Injections were made subcutaneously. The results obtained are shown in Table 4.

According to the serum tests three animals (Nos. 6, 8, and 16) were infected. Out of these three, two (Nos. 6 and 8) had aborted recently. Both animals which had aborted reacted also to the "abortin" test, but in addition to that, two more animals (Nos. 2 and 19) which were not affected, according to the serum tests, reacted to the "abortin" injection.

Conclusions.—The results with concentrated and rediluted "abortin" by subcutaneous injection are accurate, particularly when recent aborters are tested; still, inasmuch as two reactions occurred in healthy, non-infected animals, the reactions did not seem to be specific.

In this herd granular vaginitis existed at the same time to a large extent, and by the test applied, the observation and deduction could be made that this local disease

TABLE 3.
HERD No. 1.

ANIMAL NUMBER	SERUM TEST	TEMPERATURES BEFORE INJECTION AUG. 2, 1912			TEMPERATURES AFTER SUBCUTANEOUS INJECTION OF 10 C.C. OF ABORTIN AT 8:00 A.M. AUG. 2 AND 3, 1912										RESULT OF ABORTIN TEST	HISTORY
		INTER- PRETA- TION OF SERUM TEST	5:00 A.M.	6:00 A.M.	7:00 A.M.	10:00 A.M.	12:00 M.	2:00 P.M.	4:00 P.M.	6:00 P.M.	8:00 P.M.	10:00 P.M.	12:00 P.M.	2:00 A.M.		
1.....	=0.05	Positive	101.4	101.	101.	101.7	101.1	101.6	102.1	101.9	102.1	101.7	102.4	101.8	Premature calf. Never aborted.
2.....	=0.02	"	101.0	101.2	101.5	101.9	101.4	101.8	101.9	102.5	102.3	102.3	101.9	101.6	
3.....	=0	101.6	101.5	101.8	102.2	100.8	102.	102.3	102.6	103.	102.	102.2	101.7	
4.....	=0.1	101.7	101.8	101.3	102.6	101.5	102.	103.8	104.3	104.3	103.8	104.3	104.4	Aborted, 5/5/07. " 12/28/07. " 1/10/10. " 2/13/10. " 3/5/15/07. " 1/7/6/09.
5.....	=0	101.4	101.6	101.8	102.	101.	101.3	102.3	102.6	102.9	102.4	102.4	102.	
6.....	=0.02	Positive	101.1	101.	101.1	101.4	101.2	101.6	102.	102.4	102.4	101.6	101.7	101.2	
7.....	=0.05	"	101.3	101.5	101.7	102.	101.5	102.	103.	102.6	102.6	102.7	102.7	102.2	Difficult to breed.
8.....	=0	101.1	101.8	101.8	102.1	100.7	101.1	102.8	103.	102.8	103.8	102.7	102.2	
9.....	=0.02	Positive	102.	102.2	101.8	102.	101.1	101.6	102.7	102.7	103.4	103.7	103.2	102.8	
10.....	>0.005	"	101.1	101.2	101.3	101.8	101.	101.3	102.4	102.4	102.6	102.3	102.8	101.8	Difficult to breed.
11.....	=0	Positive	101.	101.1	101.5	101.6	101.1	101.3	102.1	102.1	102.	102.6	102.8	101.6	
12.....	>0.005	101.5	101.	101.8	102.3	102.3	102.	103.	103.5	103.1	102.7	101.8	101.4	
13.....	=0	Positive	101.3	102.	101.5	102.	101.8	102.2	102.2	103.	103.2	105.2	104.7	103.6	Aborted, 5/2/11. " 6/10/11.
14.....	=0.02	101.2	101.6	101.8	101.6	101.6	101.8	103.5	103.3	103.1	102.5	104.7	102.6	
15.....	=0	Positive	101.2	101.5	101.4	101.4	101.3	101.1	102.1	102.	102.2	102.6	102.4	102.6	
16.....	=0.05	"	101.	101.3	101.	102.6	101.3	101.1	102.8	103.4	103.	102.9	103.	102.1	Aborted, 12/28/07.
17.....	=0	101.1	101.2	101.	101.4	101.3	101.2	102.5	102.7	102.7	102.7	102.1	102.2	
18.....	=0.05	Positive	101.4	101.3	101.	101.0	101.4	100.9	101.8	101.9	102.7	102.1	102.2	102.8	
19.....	=0.02	"	102.4	102.6	102.4	103.	102	102.3	101.1	101.1	105.2	103.	102.4	103.	Difficult to breed.
20.....	=0	101.2	101.4	102.3	101.8	100.7	101.1	101.9	102.2	103.	103.7	101.9	102.3	
21.....	=0.02	101.3	101.4	101.8	101.0	100.7	101.2	102.6	102.7	103.5	102.1	102.0	101.8	
22.....	=0.1	101.4	101.6	101.5	101.7	101.	101.7	102.1	102.2	102.2	103.1	102.2	101.9	Difficult to breed.
23.....	=0	101.8	102.2	102.	101.0	100.4	101.2	102.4	102.8	103.1	102.2	101.9	102.2	
24.....	=0	101.0	100.4	101.2	102.9	102.	102.5	103.1	104.	104.8	101.6	102.3	104.5	
25.....	=0.1	102.3	101.9	102.6	102.9	102.	102.3	104.3	106.4	107.2	105.8	105.5	104.8	Difficult to breed.
26.....	=0	102.	102.2	102.2	103.	101.5	102.3	103.	104.	107.2	105.8	105.5	104.8	
27.....	=0	101.9	102.1	101.9	101.8	100.2	101.4	102.	102.4	102.4	103.3	105.5	101.8	
28.....	=0	Positive	102	101.8	101.8	102.4	100.3	101.9	103.5	104.9	104.3	102.6	102.5	102.	Difficult to breed.
29.....	=0.02	101.5	102.	102.5	102.	102.3	102.6	105.2	104.9	104.9	104.3	103.6	103.4	
30.....	=0	101.5	102.	102.5	103.	101.6	101.6	101.7	102.5	103.	103.4	103.6	103.4	
31.....	=0.05	Positive	101.9	102.6	102.	102.4	101.9	102.3	103.9	105.5	106.6	106.3	103.1	105.4	Difficult to breed.
32.....	=0	101.6	102.2	101.9	102.3	101.7	101.8	101.1	104.7	106.	105.	105.8	103.2	
33.....	=0	102.8	103.	103.6	102.3	102.5	102.5	102.5	103.5	103.5	103.5	103.7	103.2	
34.....	=0.02	Positive	101.8	101.7	102.	101.8	101.7	102.	102.9	102.8	102.4	102.6	103.3	102.	

TABLE 4.
HEED No. 2.

ANIMAL NUMBER	SERUM TEST	INTERPRE- TATION OF SERUM TESTS	TEMPERATURES BEFORE INJECTION AUG. 20, 1912						TEMPERATURES AFTER SUBCUTANEOUS INJECTION OF 10 C.C. OF ABORTIN AT 2.15 P.M. AUG. 20 AND 21, 1912								RESULT OF THE ABORTIN TEST	HISTORY
			9:00 A.M.	11:30 A.M.	12:30 P.M.	2:00 P.M.	4:00 P.M.	6:00 P.M.	8:00 P.M.	10:00 P.M.	12:00 P.M.	2:00 A.M.	4:00 A.M.	6:00 A.M.	8:00 A.M.			
1.....	= 0	101.9	101.8	101.5	102.5	102.	101.8	102.4	101.8	101.7	100.6	102.2	100.	100.7	Granular Vaginitis. " " " "	
2.....	= 0	102.0	102.1	102.5	103.8	102.9	101.9	103.3	103.6	102.9	102.4	101.9	101.0	101.4		
3.....	= 0	101.8	101.1	101.6	101.8	101.7	102.1	102.8	102.6	102.6	102.2	101.5	101.1	101.7		
4.....	= 0	102.3	102.8	102.1	102.4	101.8	101.8	102.9	102.8	101.9	101.2	100.6	100.4	100.7	Positive	
5.....	= 0	103.3	103.2	103.0	104.4	103.6	103.9	104.4	103.7	103.6	103.1	103.1	101.9	102.5		
6.....	= 0.05	Positive	103.6	103.5	103.6	104.4	103.2	100.3	104.6	105.2	104.4	104.3	103.6	102.4	102.2		
7.....	= 0	102.1	101.8	101.8	102.9	102.2	103.3	103.0	103.3	103.2	102.5	102.9	100.1	100.8	Positive	
8.....	= 0.05	Positive	102.6	102.1	101.8	102.7	101.8	101.5	103.3	103.1	103.1	103.4	104.6	103.2	103.1		
9.....	= 0	102.1	100.7	101.5	101.8	100.3	100.4	101.7	100.6	100.9	100.1	100.7	100.7	100.7		Granular Vaginitis. Aborted, July, 1912; Granular Vaginitis.
10.....	= 0	103.8	103.7	104.1	104.3	102.2	102.8	104.1	104.1	103.3	103.1	100.7	100.7	100.7		
11.....	= 0	102.2	101.5	102.2	103.0	102.0	102.9	103.5	103.6	103.1	102.9	103.1	102.5	102.4	Positive	
12.....	= 0	102.4	102.4	103.1	103.0	103.6	105.1	104.1	104.3	103.6	103.4	102.3	101.8	101.8		
13.....	= 0	102.7	101.9	102.3	103.1	102.1	102.8	102.9	102.1	101.7	101.8	102.1	102.1	102.1		" "
14.....	= 0	103.1	102.7	103.4	104.2	101.4	102.5	104.1	104.2	104.3	103.5	103.2	102.1	101.2		
15.....	= 0	102.5	102.1	102.4	102.9	101.1	101.4	102.4	103.7	102.4	101.4	101.4	101.1	101.2	" "	
16.....	= 0	102.4	102.1	102.4	102.9	101.1	101.4	102.4	103.7	102.4	101.4	101.4	101.1	101.1		" "
17.....	= 0.02	Positive	102.5	102.3	102.7	103.1	102.1	102.1	103.1	102.6	102.3	101.7	101.3	101.4	101.3		
18.....	= 0	102.6	102.1	102.7	103.1	101.1	101.6	101.9	102.1	101.4	100.4	100.5	100.9	100.4	" "	
19.....	= 0	102.8	102.1	102.6	102.7	102.1	102.7	102.3	102.6	103.1	103.1	104.1	103.6	103.6		Positive
20.....	= 0	102.8	102.1	102.6	102.7	102.1	102.7	102.3	102.6	103.1	103.1	104.1	103.6	103.6		
21.....	= 0	102.8	102.1	102.6	102.7	102.1	102.7	102.3	102.6	103.1	103.1	104.1	103.6	103.6	" "	

.8° F.

. F.

.2° F.

.4° F.

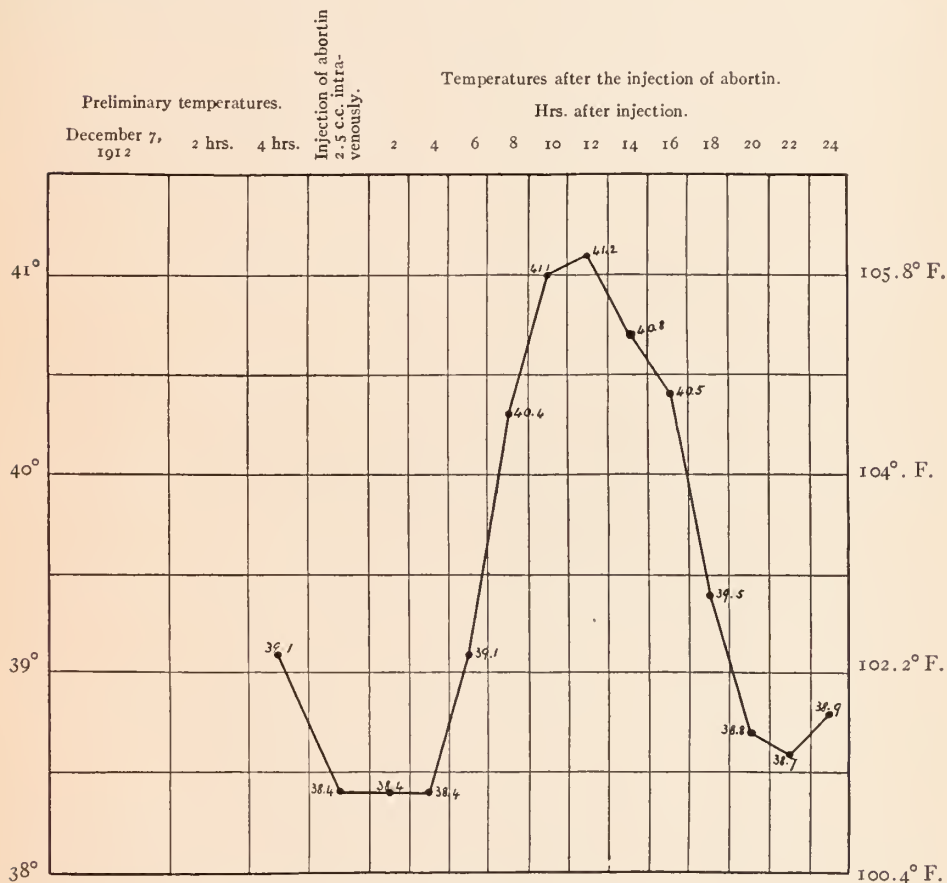
TABLE 5.
HERD No. 4.

ANIMAL NUMBER	AGE	SERUM TESTS				INTER- PRETA- TION OF SERUM TESTS	TEMPERA- TURES BEFORE INJECTION DEC. 7, 1912		INJECTION DOSE OF ABORTIN AT 1:00 P.M.	TEMPERATURES AFTER INJECTION DEC. 7 AND 8, 1912								RESULT OF THE ABORTIN TEST	HISTORY		
		1st Agglu- tination 10/30/12	1st Comple- ment Fixation	2d Agglu- tination 12/7/12	2d Comple- ment Fixation		7:00 P.M.	11:00 A.M.		4:00 P.M.	6:00 P.M.	8:00 P.M.	10:00 P.M.	12:00 P.M.	2:00 A.M.	4:00 A.M.	6:00 A.M.			8:00 A.M.	
1.....	9	>0.1	=0.005	>0.01	=0.005	Positive	38.5	38.2	10	c.c. subcutaneously	38.6	38.2	38.2	38.4	38.1	38.6	38.2	38.4	38.5	1 aborted previous to 1911.
2.....	10	=0.02	=0.02	>0.05	=0.05	"	38.6	38.0	10	"	37.9	38.5	38.5	38.0	38.2	38.6	39.8	39.3	39.0	1 aborted, 1/3/12.
3.....	9	=0.02	=0.005	>0.01	=0.005	"	38.1	38.5	2 5	" intravenously	38.4	38.2	38.4	38.1	38.5	38.7	38.6	38.7	38.4	Never aborted; O.K.; calved nor- mally 12/4/11.
4.....	9	=0.05	=0	=0.05	=0	39.0	38.9	10	" subcutaneously	39.2	38.7	39.1	39.1	39.1	38.6	38.5	38.5	38.8	Never aborted.
5.....	4	=0.02	=0.05	=0.02	=0.1	Positive	38.4	38.2	2 5	" intravenously	38.9	41.4	41.5	40.4	39.0	39.0	39.8	39.4	39.0	Positive	1 aborted, 10/17/12.
6.....	4	=0	=0	=0.05	=0.05	"	38.4	38.4	10	" subcutaneously	38.3	38.4	38.4	38.2	38.3	38.1	38.2	38.6	38.4	Never aborted.
7.....	7	=0.05	=0	=0	=0	38.3	38.1	10	"	38.1	38.3	38.3	38.3	38.1	38.2	38.5	38.7	38.7	"
8.....	6	>0.01	=0.05	>0.01	=0.005	Positive	38.3	38.1	5	"	38.3	38.5	38.5	38.4	38.4	38.6	39.1	39.0	39.1	Aborted, 8/13/09; calved, 2/11.
9.....	11	=0.05	=0	=0	=0	38.0	38.2	2 5	" intravenously	38.0	38.1	38.4	38.4	37.9	37.9	38.4	38.4	38.3	Never aborted.
10.....	16	=0.05	=0.02	>0.01	=0.05	Positive	38.2	38.5	5	" subcutaneously	38.7	38.5	39.1	38.7	38.6	38.2	38.3	38.9	38.9	"
11.....	11	=0.02	=0	=0.05	=0.1	"	38.4	38.1	2 5	" intravenously	38.2	38.1	38.5	38.2	38.1	38.0	38.3	38.5	38.3	"
12.....	13	>0.01	=0.02	>0.01	=0.005	Positive	38.9	38.2	10	" subcutaneously	38.3	38.6	38.5	38.6	39.4	40.1	40.5	40.1	39.8	Positive	1 aborted, 7/26/11.
13.....	8	>0.01	=0.005	>0.01	=0.05	"	38.7	38.3	10	"	38.6	38.6	38.8	39.1	39.0	39.1	39.3	39.4	39.5	2 abortions, 1908, 5/11/11.
14.....	4	=0	=0	=0	=0	38.6	37.9	10	"	38.1	38.4	38.6	38.0	38.0	38.0	38.2	38.7	38.6	Never aborted.
15.....	5	>0.01	=0.02	=0.02	=0.05	Positive	39.1	38.4	2 5	" intravenously	39.0	38.7	39.3	39.1	38.9	38.7	38.6	38.8	38.8	"
16.....	2	=0.02	=0.05	=0.01	=0.02	"	38.3	38.1	2 5	"	38.5	38.8	39.4	39.4	39.4	39.0	39.0	39.4	39.4	1 abortion, 9/3/12.
17.....	4	=0.02	=0.05	>0.01	=0.005	"	39.1	38.4	2 5	"	38.4	39.1	40.4	41.1	41.2	40.8	40.5	39.3	38.8	Positive	2 abortions, 7/20/10, 4/27/11.
18.....	2	=0.02	=0.05	=0.02	=0.05	"	38.3	38.3	2 5	"	38.2	38.6	40.2	40.7	40.3	39.8	39.4	39.3	40.8	"	1 abortion, 9/22/12.
19.....	2	>0.01	=0.05	>0.01	=0.1	"	38.5	38.1	2 5	"	38.2	38.0	38.6	38.5	38.4	38.7	38.6	39.0	39.1	1 abortion, 10/12/12.
20.....	3	=0.05	=0	=0.05	=0	"	38.6	38.5	2 5	"	38.9	38.1	38.0	38.7	37.9	38.3	38.4	38.3	38.7	Calved, 3/28/12 (never aborted).
21.....	5	=0.02	>0.005	>0.01	=0.005	Positive	38.4	38.6	10	" subcutaneously	39.0	38.0	38.6	39.1	39.7	40.0	39.5	38.5	38.3	Positive	Aborted, 4/10/10.
22.....	5	=0.05	=0	=0	=0	38.7	38.2	2 5	" intravenously	38.2	37.9	38.1	38.5	38.1	38.2	38.2	38.1	38.7	Never aborted.
23.....	7	=0.05	=0	=0	=0	"	38.2	38.4	2 5	"	38.8	38.4	38.3	38.5	38.6	38.2	38.6	38.4	38.4	"
24.....	3	>0.01	=0.05	=0.02	=0.05	Positive	38.3	38.5	2 5	"	39.1	38.7	39.4	38.7	40.0	40.6	40.6	39.5	39.3	Positive	"
25.....	13	=0.05	=0	=0	=0	38.9	38.4	2 5	"	38.8	38.7	39.1	39.0	38.9	38.8	38.6	38.7	39.0	Never aborted; not yet calved.
26.....	13	=0.05	=0	=0.05	=0	38.8	38.4	2 5	"	39.0	38.8	38.8	38.8	38.6	38.9	38.8	38.8	39.1	"
27.....	13	=0.05	=0	=0	=0	38.6	38.3	2 5	"	38.7	38.4	38.3	38.4	38.2	38.6	38.9	39.2	39.3	"
28.....	20	=0.05	=0	=0.05	=0	38.5	38.2	2 5	"	38.8	38.3	38.6	38.5	38.2	38.3	38.5	38.6	38.8	"
29.....	20	=0.05	=0	=0.05	=0	39.2	38.4	2 5	"	39.0	39.0	38.8	38.9	38.8	39.0	38.6	38.8	38.9	"
30.....	20	=0.05	=0	=0	=0	38.5	38.5	2 5	"	38.6	38.6	39.4	40.1	39.7	39.6	39.7	39.6	40.2	Positive	"
31.....	12	=0.05	=0	=0	=0	38.7	38.3	2 5	"	38.6	38.8	38.6	38.6	38.7	38.4	38.5	39.1	38.4	"
32.....	10	=0	=0.02	=0	=0.1	39.7	38.6	10	" subcutaneously	38.5	38.1	38.6	38.9	38.6	38.2	38.2	38.5	39.1	1 abortion, 9/12/10.

of the genital tract is not responsible for abortion. This is only a confirmation of what has been reported by Zwick and Zeller, Eber and others.

Herd No. 4.—In a herd of 32 pure-bred Guernseys, in which infectious abortion had existed for the last four years, we were able to apply the "abortin" test by somewhat different technic. The preparation used was a mixture of 8-weeks-old cultures concentrated and rediluted as previously mentioned. The application was made subcutaneously and intravenously. The results of the test are shown in Table 5.

TABLE 5a.



Cow 17, Herd 4. Aborted twice July 20, 1910 and April 27, 1911.

Abortin test December 7, 1912; Serum tests December 7, 1912.

Agglutination: < 0.01.

Complement fixation: 0.005.

Highest temperature before injection 102.4° F.

Highest temperature after injection 106.1° F.

Difference in temperature 3.7° F.

Reaction: positive.

According to the serum tests 15 animals (Nos. 1, 2, 3, 5, 8, 10, 12, 13, 15, 16, 17, 18, 19, 21, 24) were affected with *B. abortus*. Altogether 12 animals (Nos. 1, 2, 5, 8, 12, 13, 16, 17, 18, 19, 21, 32) had aborted in this herd. Out of these 12 animals seven (Nos. 5, 12, 17, 18, 21, 24, 30) reacted to the "abortin." The temperature rise varied between 1.5° and 3.1° C.

In Table 5a, a positive reaction in curve form illustrates the reaction. Out of the healthy ones only one animal (No. 30) reacted atypically, this animal being a heifer.

In Table 6, below, the results obtained by the intravenous and subcutaneous methods are shown in comparison:

TABLE 6.

INTRAVENOUSLY: 20				SUBCUTANEOUSLY: 12			
Aborters	Aborted in Last Two Years	Reactors to Serum Tests	Reactors to Abortin	Aborters	Aborted in Last Two Years	Reactors to Serum Tests	Reactors to Abortin
5	5	8	62.5 per cent	7	7	7	28.5 per cent
Healthy: 1				Healthy: none			

In comparing the results obtained by the different methods, we find, as shown in Table 6, that 62.5 per cent were diagnosed when the "abortin" was applied intravenously. Only 28.5 per cent were found to react to the diagnostic following the subcutaneous application. Out of the healthy animals one animal reacted to the intravenous method, and none to the subcutaneous method.

Conclusions.—From the "abortin" tests on 32 animals inoculated by different methods (subcutaneous and intravenous) with old concentrated cultures, we conclude that the intravenous method gives somewhat more accurate results than the subcutaneous method. Still the prevailing non-specificity of the reaction in the other tests is not eliminated by the intravenous method.

Herd No. 5.—The conclusions drawn from the experiments on Herd No. 4 warranted some further tests. An opportunity was offered to select 22 animals out of a herd of 138 pure-bred animals. These animals were tested by the intravenous and subcutaneous methods with an old concentrated, rediluted "abortin" of known antigenous properties. The results obtained are shown in Table 7.

Out of the 14 reactors (Nos. 18, 127, 128, 129, 130, 131, 72, 10, 42, 134, 135, 136, 137, 138) to the serum tests, 12 animals (Nos. 127, 128, 129, 130, 131, 72, 42, 134, 135, 136, 137, 138) had recently aborted. Thirteen animals (Nos. 7, 18, 31, 32, 127, 128, 129, 130, 131, 72, 42, 133, 138) altogether reacted to the "abortin," with a temperature rise which varied between 1.9° and 3.2° C. Two animals, following the intravenous test, showed general symptoms. Nine animals (Nos. 18, 127, 128, 129, 130, 131, 72, 42, 138) reacting to the "abortin" were also reactors to the serum tests. On the other hand, four animals (Nos. 7, 31, 32, 133) reacting to the "abortin" were, according to the serum tests, not infected with *B. abortus*. Neither had any of these animals ever aborted. Animals No. 130 and 131 gave at the first test a high agglutination

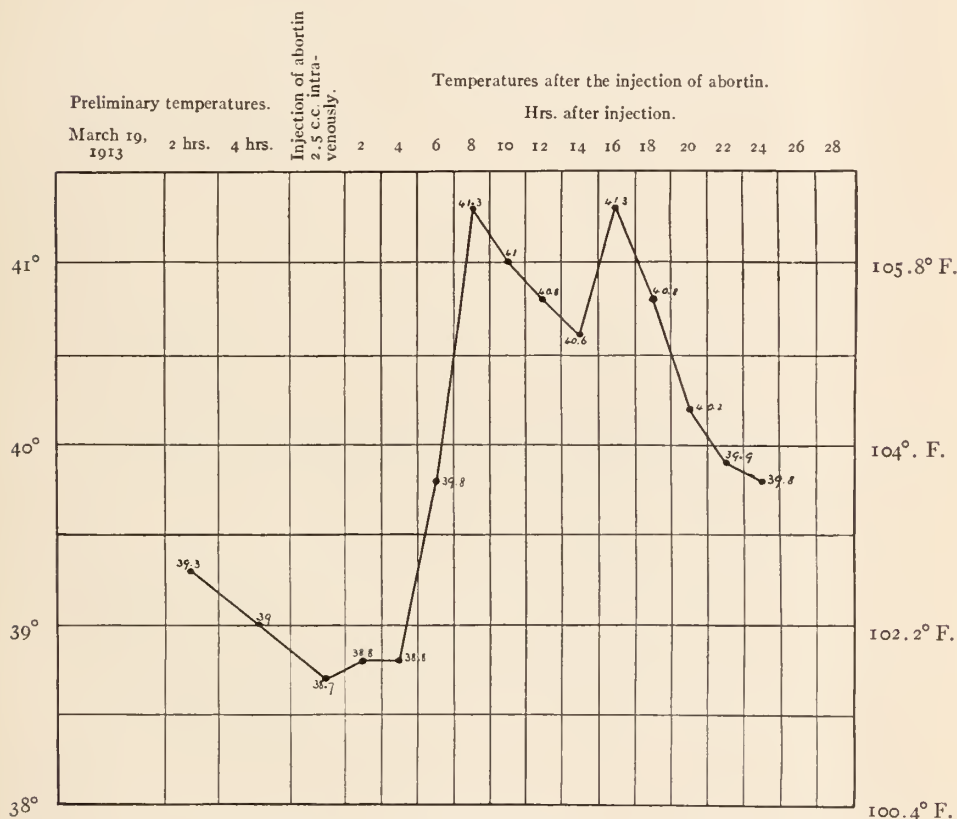
TABLE 7.
HERD No. 5.

ANIMAL NUMBER	SERUM TESTS		INTER- PRETA- TION OF SERUM TESTS	TEMPERATURES BEFORE INJECTION MARCH 19, 1913			DATE 3/19/13 Dose of Abortin Injected at 10:00 P.M.	TEMPERATURES AFTER INJECTION MARCH 20, 1913										RESULT OF THE ABORTIN TEST	HISTORY
	1st Agglu- tination 3/1/13	1st Comple- ment Fixation		5:00 P.M.	7:00 P.M.	9:00 P.M.		1:00 A.M.	3:00 A.M.	5:00 A.M.	7:00 A.M.	9:00 A.M.	11:00 A.M.	1:00 P.M.	3:00 P.M.	5:00 P.M.	7:00 P.M.		
7.....	0	=0	38.2	37.9	2.5 c.c. intravenously	38.4	38.1	38.4	38.5	38.7	39.1	39.9½	40.	39.9	40.1	Positive	General symptoms; difficult to breed.
18.....	>0.01	=0.02	Positive	38.7	38.5	" "	38.8	38.7	38.5	38.7	38.8	39.6	40.1½	40.3	40.6	40.6	"	Difficult to breed; freshened in Scotland.
31.....	>0.01	=0	38.6	38.3	" "	38.7	38.9	40.8	40.5	40.8	41.3½	39.4	39.8	40.8	39.3	"	2d calf bred; calved, 12/8/12.
32.....	0	=0	38.5	38.5	" "	38.5	38.4	38.5	38.5	38.4	38.6½	40.1	40.4	39.9	39.	"	Calved normally.
33.....	0	=0	39.2	39.7	" "
127.....	>0.01	=0.02	Positive	38.7	38.5	2.5 c.c. intravenously	38.4	38.	38.4	39.4	41.9½	40.2	40.4	40.1	40.9	40.9	Positive	Aborted, 2/4/13; 3d calf.
128.....	>0.01	>0.005	"	38.3	38.3	" "	38.7	38.3	38.5	38.3	38.3	38.4	38.9	39.6	41.0	41.0	"	" 1/29/13; 2d calf; bred, 5/21/13.
129.....	>0.01	=0.02	"	39.3	39.	38.7	" "	38.8	38.7	39.8	41.3	41.	40.8	40.6	41.3	40.8	40.2	"	Aborted, 2/4/13; 3d calf; bred, 5/19/13.
130.....	>0.01	>0.02	"	38.5	38.	" "	38.4	39.1	40.6	40.2	39.3	38.8	40.4	39.9	39.4	39.9	"	Aborted, 2/6/13; 2d calf; bred, 5/3/13.
131.....	>0.01	>0.005	"	38.6	38.	" "	38.1	38.5	38.5	40.1	41.3	41.1	41.4	41.6	"	Aborted, 12/8/12; trembling; general symptoms.
132.....	>0.01	=0.1	38.2	37.6	" "	37.9	38.2	37.7	38.4	38.4	38.4	38.1	38.4	38.7	38.3	Aborted, 1/17/13.
71.....	0	=0.05	38.2	38.3	" "	38.2	38.5	38.3	38.6	38.1	38.6	39.6	39.9	39.7	38.9	" 1910.
72.....	=0.05	=0.02	Positive	38.4	38.4	" "	38.2	38.6	38.3	38.1	38.2	38.7	39.6	40.5	39.6	39.5	Positive	" 1910.
9.....	0	0	38.5	38.1	10 c.c. subcutaneously	38.2	38.3	38.5	38.3	38.5	38.5	38.7	38.5	39.1	38.9	Calved normally.
10.....	>0.01	=0.05	Positive	38.5	38.3	" "	38.3	38.5	38.8	38.7	38.5	38.5	38.9	38.7	38.3	38.8	3d calf bred.
34.....	=0.05	38.5	38.7	" "	39.1	39.4	38.8	38.4	38.4	38.5	38.6	38.7	38.6	Calved normally, 8/13/12; difficult to breed.
42.....	>0.01	=0.02	Positive	38.5	39.5	" "	38.4	38.5	38.9	39.5	40.5	40.9	40.4	40.5	40.2	39.9	Positive	Aborted, June, 1912.
133.....	>0.01	0	38.4	38.3	" "	38.2	38.2	38.4	40.9	38.2	38.4	39.5	38.5	39.3	38.5	"	Calved, 4/15/12.
134.....	>0.01	>0.005	Positive	38.8	38.3	" "	38.3	38.7	38.9	39.1	39.2	39.	39.0	39.3	39.6	39.5	Aborted, 2/7/13; 5th calf.
135.....	>0.01	>0.005	"	38.9	37.9	" "	38.3	38.5	38.6	38.8	39.	38.9	38.9	38.9	39.7	39.4	" 2/10/13; 2d "
136.....	>0.01	>0.005	"	38.6	38.4	" "	38.2	38.3	38.5	38.7	38.1	38.5	38.7	39.1	39.7	39.8	" 2/27/13; 4th "
137.....	>0.01	>0.005	"	38.9	39.	" "	38.7	38.5	39.	38.8	38.2	38.3	38.6	38.1	39.1	38.9	" 2/25/13; 1st "
138.....	>0.01	>0.005	"	38.4	38.1	" "	38.3	38.9	39.2	39.4	39.7	40.4	39.9	40.2	40.7	40.0	Positive	" 3/1/13; 4th calf (prema- ture).

reaction, but no complement fixation. On the retest, which was carried out several weeks afterward, the animals gave a marked complement fixation reaction. Animal No. 133, a heifer just bred, is based on the high agglutination, probably in the stage of incubation, and gave a distinct positive reaction to the "abortin." This is noteworthy for further investigations, with the object of determining if it is not possible that an early infection can perhaps be more readily determined by the "abortin" test.

In comparing again the results of the different methods, intravenous and subcutaneous, we note from Table 8 that by the intravenous method 100 per cent accurate results were obtained; by the subcutaneous method on practically the same number

TABLE 7a.



Cow 129, Herd 5. Aborted February 4, 1913.
 Abortin test March 19, 1913. Serum tests March 1, 1913.
 Agglutination: <0.01.
 Complement fixation: 0.02.
 Highest temperature before injection 102.6° F.
 Highest temperature after injection 106.2° F.
 Difference in temperature 3.6° F.
 Reaction: positive.

of reactors and aborters only 18.5 per cent were accurately diagnosed by the "abortin."

On the other hand, the percentage of non-specific reactions by the intravenous method is higher than by the subcutaneous method. The percentage of failures in healthy animals by the intravenous method was 60 per cent and by the subcutaneous, 33.3 per cent.

TABLE 8.

INTRAVENOUSLY: 12				SUBCUTANEOUSLY: 10			
Aborters	Aborted in Last Two Years	Reactors to Serum Tests	Reactors to Abortin	Aborters	Aborted in Last Two Years	Reactors to Serum Tests	Reactors to Abortin
8	6	7	10 (7) (100 per cent)	6	6	7	3 (2) (18.5 per cent)
Healthy: 3 out of 5 animals (60 per cent)				Healthy: 1 out of 3 animals (33.3 per cent)			

Conclusions.—Out of 22 aborters and healthy animals tested by intravenous and subcutaneous "abortin" injections, it was shown that 100 per cent accurate results were obtained by the intravenous method. On the other hand, a rather high percentage of failures was noticed when using this method of injection of the biologic product. The results obtained show again that the "abortin" is non-specific, but that certain valuable information can be obtained by its application, particularly when the intravenous method is used.

Herd No. 6.—To prove the above statements, that the intravenous method may give valuable information, we considered it wise to test a small herd in which abortion had recently occurred, purely by the intravenous method. On April 5, 1913, 18 animals were tested. The "abortin" preparation used for this test was the same as used in Herd No. 5. The gratifying results are shown in Table 9.

According to the serum tests four animals (Nos. 13, 5, 19, 8) were infected with *B. abortus*. One animal (No. 8) had aborted two days previous to the test. All (Nos. 13, 5, 19, 8) the animals which gave positive reactions to the serum tests reacted with a marked elevation of temperature, showing differences between 1.7° and 4.6° F. In Table 9a one temperature reaction is shown in curve form. None of the healthy animals reacted to the "abortin." In comparing the results, here we note that in a herd of 18 animals, 100 per cent positive results were obtained by the intravenous method.

Conclusions.—In a small herd of 18 animals, in which abortion has been present for only a very short time, 100 per cent of positive results were obtained by the intravenous method.

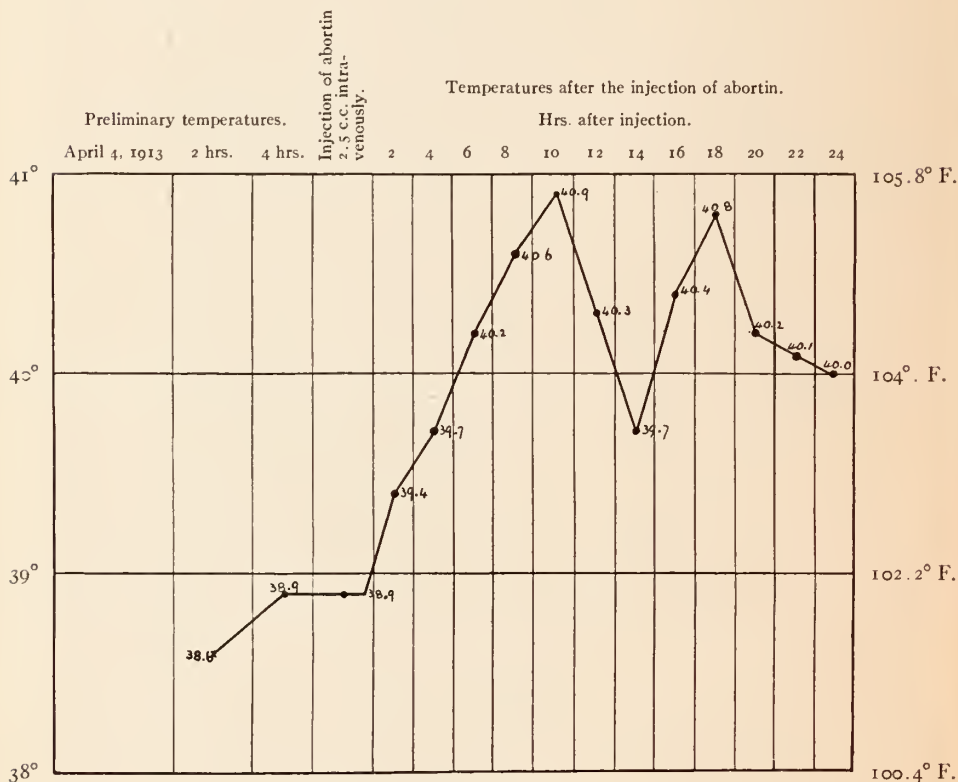
Herd No. 7.—The high percentage of failures in absolutely healthy animals which deprived the preceding "abortin" test of its specificity was thought to be perhaps overcome by purifying the "abortin," and for this reason a herd was tested with precipitated and "plain" concentrated "abortin," prepared as explained and discussed in the introduction. The herd selected for this purpose consisted of 69 animals, in which, according to complete records, 31 animals had aborted. The test was made on May 13, 1913, and the results are shown in Table 10.

TABLE 9.
HERD No. 6.

ANIMAL NUMBER	SERUM TESTS				INTER- PRETA- TION OF SERUM TESTS	TEMPERATURES AFTER INTRAVENOUS INJECTION OF 2.5 C.C. OF ABORTIN AT 9:00 P.M. APRIL 5, 1913										RESULT OF ABORTIN TEST	HISTORY	
	TEMPERATURES BEFORE INJECTION APR. 4, '13																	
	1st Agglu- tination 2/25/13	1st Comple- ment Fix- ation	2d Agglu- tination 4/4/13	2d Comple- ment Fix- ation		6:00 P.M.	8:00 P.M.	12:15 A.M.	4:15 A.M.	6:15 A.M.	7:30 A.M.	9:30 A.M.	11:30 A.M.	12:30 P.M.	1:30 P.M.			3:30 P.M.
2	= 0.02		= 0.05			101.2	101.0	101.8	101.1	101.2	102.1	102.3	101.5	101.1	101.1	101.2		
3	= 0.05		= 0.02			102.1	101.8	101.5	101.6	102.1	102.3	101.8	102.1	101.3	101.2	101.4	101.6	
4	= 0.05		= 0.02		Positive	101.4	102.3	101.4	101.8	102.5	103.8	104.1	103.5	102.6	102.2	101.6		Positive
5	> 0.01	= 0.005	> 0.01	= 0.005		101.4	102.3	101.4	101.4	101.7	101.6	102.1	101.1	100.8	101.2	101.8		
6	= 0.02		= 0.02			101.1	101.8	101.2	101.4	101.4	101.7	101.6	102.1	101.1	100.8	101.1		
7	= 0.02		= 0.02		Positive	101.1	101.4	100.8	101.3	102.3	102.4	102.2	101.1	100.8	101.1	101.6		
8	> 0.01	= 0.05	> 0.01	= 0.02		101.4	101.8	102.8	103.2	104.3	105.1	105.6	104.2	103.3	104.6	105.4		
9	= 0.02		= 0.02			101.5	101.3	101.5	101.8	101.7	102.3	102.4	101.3	101.1	101.4	101.1		Heifer breed.
10	= 0.05		= 0.05			101.8	102.1	101.2	101.5	102.1	102.3	102.4	101.3	101.1	101.2	101.2		
11	= 0.05		= 0.05			101.3	101.6	101.5	101.2	101.7	102.1	102.3	102.4	101.3	101.1	101.4		A. 4/2/13.
12	= 0.02		= 0.02			101.6	101.4	102.2	101.4	102.1	101.8	102.3	101.1	101.3	101.1	101.5		
13	> 0.01	= 0.02	> 0.01	= 0.02	Positive	101.2	101.4	101.4	101.4	104.8	104.4	105.4	105.2	105.8	105.1	104.2		Heifer breed.
14	= 0.01		= 0.02			101.4	101.8	101.4	102.2	102.5	102.1	101.6	101.8	101.2	101.5	101.4		
15	= 0.02		= 0.02			101.1	101.8	101.6	101.8	101.4	101.6	101.2	101.7	101.3	100.8	101.5		
16	= 0.01		= 0.02			101.2	101.6	100.9	101.2	102.1	101.4	101.8	102.8	102.3	101.6	101.8		
17	= 0.05		= 0.02			101.4	101.5	101.1	101.2	101.8	102.3	102.2	102.3	101.6	101.6	101.6		
18	= 0.05		= 0.05			102.3	102.8	102.1	101.9	102.1	102.2	102.8	102.6	101.7	102.2	101.8		
19	> 0.01	= 0.02	> 0.01	= 0.02	Positive	102.8	102.5	102.4	101.6	105.2	105.4	105.8	105.7	105.5	104.8	103.6		Heifer breed.

According to the serum tests 26 animals (Nos. 7, 8, 11, 13, 17, 20, 21, 22, 26, 28, 35, 38, 43, 101, 103, 106, 110, 111, 113, 114, 124, D, B, C, E, A) were still showing immune bodies in the serum. Twenty animals (Nos. 4, 7, 11, 22, 23, 26, 28, 29, 35, 43, 44, 50, 57, 106, 111, D, B, C, E, A) reacted to the "abortin" test, out of which 5 animals (Nos. 4, 29, 44, 57, 50) were healthy. Only 15 serum reactors (Nos. 7, 11,

TABLE 9a.



Cow 8, Herd 6. Aborted April 2, 1913.

Abortin test, April 4, 1913.

Agglutination: <0.01.

Complement fixation: 0.02.

Highest temperature before injection 101.4° F.

Highest temperature after injection 105.6° F.

Difference in temperature 4.2° F.

Reaction: positive.

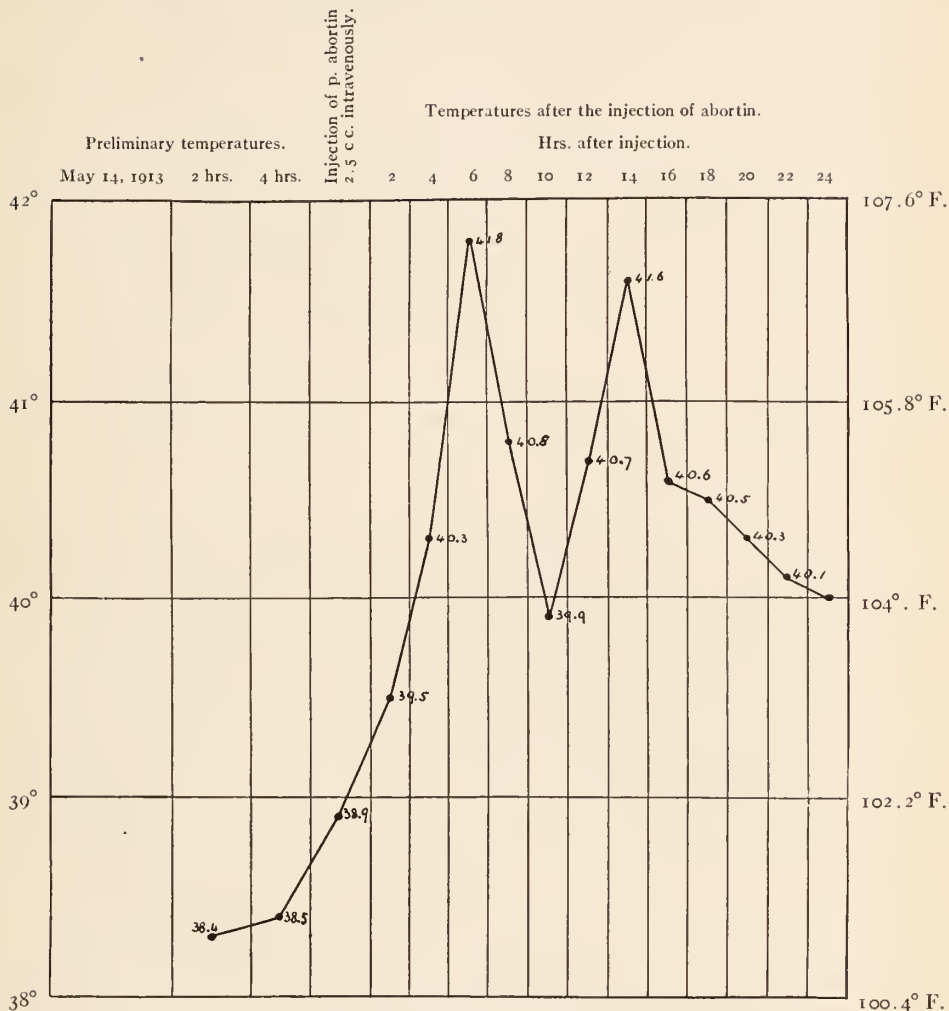
22, 23, 26, 28, 35, 43, 106, 111, D, B, C, E, A) actually reacted to the "abortin." In Table 10a one of the reactions is shown in curve form.

The temperature with the precipitated "abortin" showed differences between the preliminary and the test temperatures from 2.2° to 5.2° F., average 3.91° F. With

TABLE 10.
HERD No. 7.

ANIMAL NUMBER	AGE YEARS	SERUM TESTS				INTER- PRETA- TION OF SERUM TESTS	TEMPERA- TURES BEFORE INJECTION MAY 13, 1913		INJECTION	TEMPERATURES AFTER INJECTION MAY 14, 1913										RESULT OF THE ABORTIN TEST	HISTORY		
		1st Agglu- tination 7/10/12	1st Comple- ment Fixation	2d Agglu- tination 4/21/13	2d Comple- ment Fixation		4:00 P.M.	6:00 P.M.		Dose of Abortin at 7:00 P.M. Precipitated	12:00 P.M.	2:00 A.M.	4:00 A.M.	6:00 A.M.	8:00 A.M.	10:00 A.M.	12:00 N.	3:00 P.M.	5:00 P.M.				
2.....	6	=0.05	=0	=0.05	=0	101.4	101.4	2.5 c.c. intravenously	101.3	101.8	101.4	101.5	101.8	101.	101.	Aborted, 7/24/09; calved, 8/27/12 (calf died); Granular Vaginitis; difficult to breed.			
3.....	10	=0.05	=0	=0.05	=0	101.2	101.2	"	101.2	101.9	101.6	101.6	101.	101.4	101.2	Calved, 3/23/12; difficult to breed.			
4.....	8	=0.02	=0	=0	=0	101.	101.2	"	101.	101.2	102.	103.8	105.6	104.6	103.	100.9	Positive	" 2/28/13.			
5.....	11	=0.05	=0	=0.05	=0	102.3	102.8	"	102.5	103.1	103.	102.	101.8	102.	101.8	" 7/9/12; due to calf 6/20/13.			
6.....	5	=0.02	=0.1	=0.05	=0	101.2	101.2	"	101.2	101.2	101.8	101.9	101.5	101.8	102.	Aborted, 7/4/09; calved, 12/20/12.			
7.....	5	>0.01	=0.02	=0.01	=0	Positive	101.	101.5	"	102.2	101.6	102.2	102.5	103.2	102.2	104.8	102.	Positive	" 8/7/09; last calf, 3/7/13.			
8.....	5	>0.01	=0.05	=0.05	=0.05	"	101.8	101.9	"	102.5	103.	104.8	105.8	102.8	101.3	103.3	103.	Never aborted; due 7/10/13; last calf, 6/29/12.			
9.....	5	=0.02	=0	=0.02	=0	102.	101.8	"	Not tested, due to calf 6/9; last calf, 3/8/12.			
10.....	12	=0.05	=0	=0.05	=0	101.	101.2	"	101.7	101.2	101.3	101.8	101.8	100.8	101.2	Calved, 11/28/12.			
11.....	9	>0.01	=0.02	>0.01	=0.02	Positive	101.2	102.2	"	102.8	104.8	105.5	105.	104.9	104.9	105.	104.3	Positive	Aborted, 4/6/08; calved, 3/7/09 (calf died); last calf, 9/30/12.			
12.....	9	=0.02	=0	=0.05	=0	101.3	102.1	"	101.8	101.8	102.	102.2	101.9	101.7	102.	Aborted, 3/6/07; last calf, 8/1/12.			
13.....	12	>0.01	=0.02	=0.02	=0.05	Positive	101.2	101.7	"	101.8	101.6	101.4	102.	102.	101.3	101.8	" 11/8/08; calved, 3/12/13.			
14.....	9	=0.02	=0	=0.01	=0	101.5	101.5	"	101.4	102.2	100.5	101.8	101.6	101.2	100.9	" 10/14/07; calved, 12/21/12.			
15.....	7	=0.05	=0	=0	=0	101.1	101.2	"	101.7	102.2	102.2	102.8	103.2	102.2	103.4	103.2	Calved, 3/18/13.		
16.....	12	=0.02	=0	=0.05	=0	102.	101.8	"	101.4	101.8	102.1	102.	102.2	100.9	101.4	Aborted, 3/24/08; last calf, 3/14/13.			
17.....	12	=0.02	=0	>0.01	=0.005	Positive	102.	101.7	"	102.	102.	101.4	102.2	102.4	101.	102.3	Last calf, 7/17/12.			
18.....	11	=0.02	=0	=0.05	=0	101.8	101.8	"	101.7	101.8	101.8	101.1	101.6	101.2	101.6	Calved, 2/12/13.			
19.....	6	=0.05	=0	=0.05	=0	101.5	102.4	"	103.2	102.	102.7	102.5	102.7	101.	103.	102.3	Aborted, 10/7/09; last calved, 3/15/13.		
20.....	5	>0.01	=0.005	>0.01	=0.02	Positive	102.4	102.1	"	102.2	102.	101.6	102.1	102.6	102.3	103.1	103.3	Calved, 5/12/12; difficult to breed.		
21.....	4	=0.02	=0	"	101.8	102.1	"	101.2	101.8	101.8	102.4	102.	101.2	102.4	103.	104.4	Positive	Calved, 7/18/12.		
22.....	6	=0.02	=0.05	=0.05	=0	101.5	101.3	"	101.6	103.	103.8	103.6	102.9	103.4	104.6	103.1	Aborted, 1/10/10; calved, 12/20/12.		
23.....	6	=0.02	=0	=0.05	=0	102.	102.4	"	102.2	101.9	101.8	102.	101.8	101.9	101.6	" 9/10/09; calved, 9/14/12 (calf died).			
24.....	6	=0.02	=0	102.1	102.2	"	102.1	102.	102.	102.7	102.5	99.	101.	Aborted 10/7/08; calved, 4/21/13.			
25.....	6	=0.02	=0	101.3	101.3	"	101.8	101.8	101.4	101.6	101.8	101.8	101.8	" 4/28/09; calved, 12/7/12.			
26.....	6	>0.01	=0.005	=0.02	=0.005	Positive	101.8	101.2	"	102.2	102.8	102.	104.	103.3	103.5	104.2	Positive	Never aborted; calved, 9/3/12 (calf died).		
28.....	4	>0.01	=0.005	>0.01	=0.005	"	101.8	101.8	"	105.2	106.	104.	102.4	104.2	103.5	105.2	"	Aborted, 7/19/11; calved, 9/9/12 (4 months delivered).		
29.....	4	>0.01	=0	101.8	101.8	"	103.4	106.2	105.	102.3	102.4	102.1	105.2	102.6	Aborted, 8/4/11; calved, 9/17/12.		
30.....	3	=0	=0	101.8	101.9	"	101.4	101.8	101.4	102.2	101.7	101.4	101.6	Calved, 1/13/13.		
34.....	4	=0.05	=0	=0.02	=0	101.2	101.	"	101.2	101.2	101.	101.3	101.6	101.2	102.6	Last calved, 3/13/13.		
35.....	4	>0.01	=0.005	>0.01	=0.005	Positive	101.4	101.3	"	104.	105.8	105.	103.4	104.4	104.4	105.4	104.	Positive	Aborted, 7/24/11 and 10/24/10; last calved, 7/1/12.	
36.....	3	=0.02	=0.1	=0.05	=0	101.	101.6	"	101.2	101.	101.2	102.	101.3	100.6	100.8	Calved, 7/16/12.		
37.....	3	=0.02	=0	=0.02	=0	101.5	101.6	101.2	101.2	101.3	102.	101.8	101.4	101.	" 7/28/12.		
38.....	3	>0.01	=0.02	=0.05	=0.02	Positive	101.3	102.2	2.5 c.c. intravenously	102.2	102.	101.6	101.9	102.3	101.6	101.8	Due to calf, 6/20/13.		
39.....	3	>0.05	=0	=0.05	=0	101.4	101.6	"	101.1	101.5	101.8	102.1	101.7	100.8	101.2	Aborted, 4/15/11; due 6/25/13—calved, 5/14/12.		
40.....	3	>0.05	=0	=0.05	=0	101.5	101.6	"	104.	101.6	101.2	102.	101.8	100.7	101.	Calved, 5/6/12 (7/5/13).		
41.....	2	=0.02	=0	101.1	102.	"	102.	102.2	102.	102.	101.9	102.5	102.	" 12/19/12 (1st).		
42.....	2	=0.05	=0	101.	101.6	"	101.8	101.8	101.8	102.1	101.9	101.3	101.8	" 12/11/12 (1st).		
43.....	2	=0.02	=0.005	Positive	101.9	101.8	"	103.2	104.3	105.5	105.2	107.	104.5	105.8	105.6	Positive	Never aborted; calved, 1/26/13 (calf died) (1st).
44.....	2	=0.05	=0	101.6	101.5	"	102.	104.4	106.	103.9	102.2	102.	101.5	"	Never calved (due July).	
Heifer 45	2	=0.01	=0	=0.02	=0	101.	101.2	"	101.	101.2	101.3	102.	102.1	101.6	102.2	"	" " " "	
" 47	1 1/2	=0.05	=0	101.6	101.9	"	101.8	102.	101.8	101.9	101.9	102.	101.9	"	" " " "	
" 48	2	=0.01	=0	=0.05	=0	101.8	102.	"	102.	102.	102.1	102.2	102.	102.	101.4	"	" " " "	
49.....	2	=0	=0	101.6	102.2	"	101.8	102.	101.8	102.	101.8	101.9	101.6	"	" " " "	
50.....	2	=0.02	=0	101.8	102.4	"	102.4	103.2	103.7	104.	105.1	105.2	105.8	105.3	Positive	" " " "
51.....	2	=0.05	=0	=0.05	=0	101.6	102.2	"	101.2	101.3	101.5	101.8	101.4	101.2	101.3	"	" " " "	
52.....	1 1/2	=0.02	=0	=0.05	=0	101.6	101.4	"	101.8	101.2	101.5	101.5	101.8	101.3	101.6	"	" " " "	
53.....	1 1/2	=0.02	=0	101.4	101.5	"	101.2	101.	101.	101.5	101.3	101.	101.	"	" " " "	
54.....	1 1/2	=0.02	=0	=0.02	=0	101.2	101.4	"														

TABLE 10a.



Cow D, Herd 7. Aborted July 2, 1912.

Abortin test May 14, 1913; Serum tests May 14, 1913.

Agglutination: <0.01.

Complement fixation: <0.005.

Highest temperature before injection 102.0° F.

Highest temperature after injection 107.2° F.

Difference in temperature
5.2° F.

Reaction: positive.

the plain "abortin," differences from 2.8° to 5.1° F., an average of 3.76° F., were recorded. Comparing the results obtained with the ordinary and the precipitated "abortin" in this herd by the intravenous method, the following interesting results, as shown in Table 11, are of value.

TABLE 11.
COMPARISON OF ORDINARY WITH PRECIPITATED "ABORTIN" TEST IN THE SAME HERD.
Application: Intravenously.

PLAIN "ABORTIN": 33 ANIMALS				PRECIPITATED "ABORTIN": 36 ANIMALS			
Aborted for the Last Five Years	Reacted to Serum Tests	Aborted in Last Two Years	Reacted to Abortin	Aborted for the Last Five Years	Reacted to Serum Tests	Aborted in Last Two Years	Reacted to Abortin
9	10	4	3, or 30 per cent (6, or 60 per cent)	21	16	14	14, or 87.5 per cent
Out of 20 healthy animals reacted: 3, or 15 per cent				Out of 20 healthy animals: 2, or 10 per cent			

By means of the precipitated "abortin," out of 36 animals 87.5 per cent were diagnosed correctly, with the "plain abortin" only 30 per cent were detected to be affected with *B. abortus*. Each lot of the non-infected animals inoculated with "plain" or "precipitated abortin," respectively, showed the following differences: The plain "abortin" gave 15 per cent failures; the precipitated "abortin" gave only 10 per cent failures in non-infected animals or bovines which did not show any immune bodies in the blood serum.

Conclusions.—In a herd of 69 animals, by means of the precipitated "abortin" and the intravenous method, 87.5 per cent of the animals infected with *B. abortus* could be diagnosed. In using a "purified abortin," the "non-specific reactions" can, to a certain extent, be reduced, but it has to be proven by further tests if this observation is a constant one. So far it does not seem possible to eliminate this non-specificity of the reaction.

APPENDIX.

Herd No. 3.—As explained above, "abortin" is distributed commercially, and it was considered advisable to test this preparation for its practical value. It can be mentioned here that this commercial preparation had no antigenic properties whatever, neither by animal inoculations nor by using it as an antigen in the complement fixation test. A herd, consisting of 61 animals, in which infectious abortion had existed for the last four years, was injected with this preparation, according to the instructions given by the manufacturer, the dose being 4 c.c. subcutaneously. The laboratory number of this preparation was 8815, the date of preparation was June 15, 1912. The results obtained are shown in Table 12.

Conclusions.—The commercially prepared and distributed "abortin" solution cannot be recommended for tests, because it has no antigenic properties, and the non-specificity of the preparation is also evident.

Conjunctival tests.—All the animals of Herd No. 7 were also tested by the "ophthalmic test," 2-4 drops of a 5 per cent solution of precipitated "abortin" in saline solution were instilled into the conjunctival sac as customary for such tests. The readings, made from the tenth hour on, revealed no reactions.

Conclusions.—The conjunctival test cannot be used for the diagnosis of infectious abortion.

Summary of conclusions.—In comparing the result obtained by Belfanti, and Zwick and Zeller with ours (see Table 13), we cannot blame these investigators for condemning the "abortin," as the results are certainly not encouraging. Still we do not take such a skeptical view concerning this preparation.

From the above stated experiments with "abortin" on 209 animals with our own preparations, we learned that the product permits a conclusive diagnosis in 59.3 per cent of the cases of infection, if the serum reaction is taken in consideration for comparison. In healthy animals about 29.1 per cent failures are recorded, or only 70.9 per cent of the "abortin" reactions are supported by positive serum reaction. The reactions are mostly typical, but only when a "purified precipitated abortin" is used. The failures in the healthy animals can be reduced as far as 10 per cent when this preparation is used. The injections of the biologic product should always be made intravenously; the dangers of general anaphylactic symptoms are very small in number. In recently infected herds the results are better than in old infected ones. In several instances, also, aborters reacted to the "abortin" test when there were no immune bodies in the serum of the tested animals.

We cannot support the view of Giltner that the "abortin" has a certain immunizatoric effect against natural infection, as some heifers injected with our best "abortin" have since become infected and have aborted.

SUMMARY.

The serum tests, agglutination and complement fixation, are the most reliable methods to determine the existence of infectious abortion in a herd, and to detect the bovines which are or have been infected with *B. abortus*.

The "abortin" test in the form and with the preparation recommended by the English Commission is unreliable and misleading. Encouraging results are obtained with a precipitated

TABLE 12.
HERD No. 3.

[illegible]

purified "abortin" by intravenous application. The reaction is not absolutely specific as a high percentage of healthy animals react to the injection of "abortin" products. This non-specificity is more frequently observed with an ordinary plain "abortin" than with our purified product.

By means of the "abortin" test we cannot decide whether an animal has been recently infected and will abort, or whether it is recovering from an invasion with *B. abortus*.

TABLE 13.

NO. OF HERDS	NO. OF ANIMALS IN HERD	NO. OF ABORTERS		TOTAL NO. OF REACTORS TO SERUM TEST		NO. OF REACTORS ABORTING TO SERUM TEST		NO. OF PREMATURE CALVES		NO. OF ANIMALS DIFFICULT TO BREED		REACTORS BUT NOT ABORTED		NO. OF ANIMALS REACTED TO ABORTIN EITHER WAY		NUMBERS OF REACTORS TO ABORTIN			NO. OF ABORTERS REACTED TO ABORTIN COMPARED WITH ABORTUS		NO. OF HEALTHY OUT OF REACTORS		NUMBER REACTED			INJECTED	
																	Compared with Serum Reactors	Compared with Abortin Reactors					Subcutaneously	Intravenously	Precipitated	Subcutaneously	Intravenously
			Percentage		Percentage		Percentage		Percentage		Percentage		Percentage		Percentage		Percentage	Percentage		Percentage		Percentage					
Herd No. 1. . . .	49	12	24.5	23	46.9	10	43.5	2	4	2	4	13	56.5	13	26.5	6	26.0	46.1	3	21	7	53.8	13			49	
Herd No. 2. . . .	19	2	10.5	3	15.7	2	66.6					1	33.3	4	21.8	2	66.6	50.0	2	50	2	50	4			19	
Herd No. 4. . . .	32	12	37.5	15	46.8	11	73.3					4	26.3	7	21.8	0	40.0	85.5	5	71.4	1	14.2	2	5		12	20
Herd No. 5. . . .	22(138)	14	63.6	14	63.6	12	85.7			1	4.5	2	14.3	13	59	9	65.0	69.2	8	61.5	4	30.7	3	10		10	12
Herd No. 6. . . .	18	1	5.5	4	22.2	1	25					3	75	4	22.2	4	100	100	1	25	0	0		4			18
Herd No. 7. . . .	69	31	44.9	26	37.6	17	65.4					9	34.6	20	28.9	15	57.6	75	12	60	5	25		20	14		69
Total. . . .	209	72	31	85	38.8	53	59.7	2	4	3	4.25	32	40	61	30.1	42	59.3	70.9	31	44.4	19	29.1	22	39	14	90	119
Commercial (Herd 3). . . .	61	7	11.4	9	14.7	3	33.3	1	1.6			6	66.6	2	3.49					12	100		2			61	
Belfanti. . . .	19	2	10.5	10	52.6									7	36.8	7	70	70		10.5		33.3					
Zwick and Zeller*. . . .	5	4	80	4	80	4	100					1	25	0	0	0	0	0									
	39	8	20.5	24	61.5	7	29.1					17	70.9	3	7.6	2	8.3	66.6	0	0	1	33.3					

* Selection from the report smade by the writers, *op. cit.*, p. 105.

THE PRODUCTION IN MONKEYS OF ANTIBODIES FOR HUMAN CORPUSCLES.*

LUDVIG HEKTOEN.

(From the Memorial Institute for Infectious Diseases, Chicago.)

In the course of certain experiments on measles and other diseases in the monkey¹ the opportunity was used to follow the development in the monkey of antibodies to human blood corpuscles. Uhlenhuth and Weidanz² assert that the injection of human serum in monkeys (*Cercopithecus fuliginosus* and *Macacus rhesus*) results in the formation of specific precipitins, but I am not aware that any observations have been recorded on the development in the monkey of antibodies to human corpuscles. My observations were made on five monkeys (*Macacus rhesus*) injected with human blood as follows: Monkey 1 received 3 c.c. of human blood into the heart, Monkey 5, 3 c.c. into the peritoneal cavity, Monkeys 7 and 8, 30 c.c. into the heart, and Monkey 9, 30 c.c. into the peritoneal cavity. The forms of antibody action detected were agglutination and, in the case of Monkey 5, opsonification. The agglutinative mixtures were in quantity 0.6 c.c.; of this, 0.2 c.c. was a 5 per cent suspension of washed human corpuscles, the rest, monkey serum and salt solution. The opsonic mixtures were made in the same way with the addition of washed monkey leukocytes obtained from exudates produced by the injection of suspensions of aleuronat into the pleural cavity, the total quantity here also being 0.6 c.c. The smears were made after an incubation at 37° C. for one hour.

There was no phagocytosis in similar mixtures in which human leukocytes were substituted for leukocytes from the monkey.

The heated serum of these monkeys in quantities of 0.2 c.c., complemented either with fresh monkey serum or with guinea-pig

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¹ Hektoen and Eggers, *Jour. Am. Med. Assn.*, 1912, 47, p. 1833.

² *Handbuch der Technik und Methodik der Immunitätsforschung*, 1909, 2, p. 781.

Since writing this article Berkeley reports that *Macacus rhesus* and certain Java monkeys immunized with human, horse, or dog serum do not form antibodies demonstrable by either the precipitin reaction or the reaction of fixation. (Hugh K. Berkeley, *Univ. Cal. Publications in Pathology*, 1913, 2, p. 105).

serum (0.0125 c.c.), did not cause any lysis of human corpuscles (0.2 c.c. of a 5 per cent suspension). No tests for precipitin were made.

The results as to agglutination are given in Table 1.

TABLE 1.

AGGLUTINATION OF HUMAN CORPUSCLES BY THE SERUM OF MONKEYS INJECTED WITH HUMAN BLOOD.

Days after Injection	Monkey 1. 3 c.c. of Human Blood in Heart	Monkey 5. 3 c.c. of Human Blood Intraper.	Monkey 7. 30 c.c. of Hu- man Blood in Heart	Monkey 8. 30 c.c. of Hu- man Blood in Heart	Monkey 9. 30 c.c. of Hu- man Blood In- traper.
2			0	0	0
3			0	0	0
4			0	0	0
5			0	0	0
6			0	0	0
7			6	12	6
8	192	48	24	12	6
9	384	48	24	12	24
10				12	12
11	384	48	12	12	12
12	384	48	12	12	12
13	384	48	0	12	12
14	384	48	0	0	12
15	384		0	0	0
16	192		0	0	0
17		48	0		0
18					
19		48			

The figures represent the highest dilution of the monkey serum giving agglutination.

In some instances the same results were obtained with corpuscles from five different persons of different isoagglutinative groups, showing that the agglutination did not depend on any individual peculiarity of the corpuscles.

Inspection of Table 1 shows that the highest agglutinative strength developed in the serum of Monkey 1, which received 3 c.c. of human blood into the heart. Unfortunately the day of the earliest appearance of agglutinin in the blood was not determined in this case. In the other animals the agglutinin content was not nearly so high and in those injected with 30 c.c. of human blood the period of latency was rather long, the first appearance of agglutinin being on the seventh day. This result is in harmony with the experience that, in general, large doses of antigen do not necessarily give the largest output of antibodies.

In Monkey 5 the opsonin strength of the serum was tested on the same day as the agglutinin; the extinction occurred uniformly in dilutions of 1 to 12.

The results of these observations indicate that in the monkey, injections of human blood stimulate the formation of agglutinin and opsonin for human corpuscles, these antibodies describing the same sort of a curve, tho of small range, as other antibodies after the injection of a single dose of antigen. If we may judge from just one or two instances, the injection of monkeys with comparatively small quantities of human blood gives a larger response than injections of large quantities.

The fact that monkeys respond in this way to human blood might be taken advantage of in differentiating between human and monkey blood, but self-evidently this would be possible only under very special conditions, so that the method would seem to have an extremely limited practical significance.

REPORT OF SOME EXPERIMENTAL WORK ON THE
USE OF METHYLENE BLUE AND ALLIED DYES
IN THE TREATMENT OF TUBERCULOSIS.*

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF
TUBERCULOSIS. VII.

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A preliminary report of experiments in the vital staining of tubercles was recently published by me,¹ showing which of the 20 or more dyes used penetrated the tubercle, which stained the tubercle bacillus, and which had any bactericidal power over that organism. In these earlier experiments, it was found that methylene blue penetrated the tubercle fairly well, that it stained the tubercle bacillus sharply and had *in vitro* some bactericidal power over these organisms and a considerable inhibitory action over their growth. These data being settled, it seemed worth while to try to determine how much, if any, therapeutic influence this dye had over the disease in question.

Methylene blue was one of the first dyes used *intra vitam*, as well as one of the earliest used in therapeutics. Its use as a vital or supravital stain of the nervous system, first introduced by Ehrlich,² and later developed by numerous neurological investigators in the effort to increase our knowledge of the structure of the nervous system and especially of the peripheral nervous system, is too well known to need further mention.

It was early used also as a vital stain for the lower plant and animal life. Hieronymus³ in 1893 succeeded in staining with methylene blue the vacuoles of yeast cells while they were still alive, and Przesmycki⁴ in 1894 with the same dye stained cell granulations in protozoa. Ruzicka⁵ in 1904 stained bacteria and their intracellular granules with methylene blue and also fungi and leukocytes of different animals, observing in the stained cells extrusion of processes, division of granules, and other changes which he interprets as indicating the continued life of the cell. Methylene blue is, however, more or less toxic to these lower forms of plant and animal life and for this reason was suggested as early as 1880 as an internal antiseptic. Ruzicka⁶ notes that in experiments in which he used both neutral red and methylene blue, part

* Received for publication October 1, 1913.

¹ *Jour. Infect. Dis.*, 1913, 12, p. 68.

² *Deutsch. med. Wchnschr.*, 1886, 12, p. 49.

³ *Deutsch. bot. Gesellsch.*, 1893, 11, p. 176.

⁴ *Biol. Centralbl.*, 1894, 14, p. 620.

⁵ *Ztschr. f. allg. Physiol.*, 1904, 4, p. 141.

⁶ *Op. cit.*

of the granules in the living cells took the red stain and part the blue, while the nucleus was unstained. As soon as the cell began to lose its vitality, the nucleus began to stain blue and finally, in the dead cell, nucleus and all granules were blue. In 1891, Einhorn¹ showed that the urine of patients who had been treated with methylene blue was sterile and remained sterile for three weeks. In 1900, Chaleix-Vivie,² because of the common use of the dye in gynecologic work, tested its bactericidal action on *Staphylococcus albus*, streptococcus, *B. coli*, *B. subtilis*, and other bacteria most frequently found in the utero-vaginal tract, and found that only *B. subtilis* retained its vitality after 24 hours' exposure to a dilute solution of the dye. In 1913, Churchman³ partially verified this work, at least so far as the inhibitory action of methylene blue was concerned, since he found, by his divided plate method, a certain selective action of methylene blue, tho not always uniform.

It was perhaps a natural result of the observation of the specific staining action of methylene blue on the nervous system and especially on the nerve fibers that this dye was introduced into therapeutics by Ehrlich and Lippmann⁴ in 1891 as an analgesic. The *schmerzstillende Wirkung* ascribed to the dye by these observers led to its use in neuralgias, nervous headaches, muscular rheumatism, epilepsy, migraine, psychoses, pleuritic pains of tuberculosis, etc.

It was found by Müller⁵ and also by Elsner⁶ that the dye was largely excreted by the kidneys, Elsner finding from 40 to 50 per cent of the total intake excreted by the kidneys in the first three days, 4 to 8 per cent by the intestines, while the rest could not be recovered or accounted for. Müller's figures were a little higher, as he recovered about 70 per cent of the total intake. Since the renal excretion begins very early, the urine becoming bluish green in about 15 minutes after the ingestion of the dye, it was thought that both its bactericidal and its analgesic power might be invoked in the treatment of diseases of the kidney, bladder, and urethra. Accordingly ordinary pyogenic infections and also gonorrheal infections of the genito-urinary apparatus have been treated more or less effectually with methylene blue. It has also been shown to have some diuretic action and its rapid excretion makes it valuable in testing renal permeability and function. Dysentery and other infections of the digestive tract have also been treated with methylene blue.

In line with the bactericidal action of the dye, Ehrlich and Gutmann⁷ in 1891 announced that methylene blue, which stained malarial plasmodia very well, had been used by them in the treatment of malaria; they showed that it relieved the symptoms of malaria and eventually caused the disappearance of the plasmodia from the blood. Other workers have used the dye in this disease with greater or less benefit and, while it does not replace quinin, it may be used with it or after it has failed to conquer the disease.

In 1891, about the time when Ehrlich and Lippmann reported the analgesic action of methylene blue, Einhorn⁸ began to use the dye in the treatment of some eight cases

¹ *Deutsch. med. Wchnschr.*, 1891, 17, p. 620.

² *Compt. rend. Soc. de biol.*, 1900, 5, p. 242.

³ *Jour. Exper. Med.*, 1913, 18, p. 187.

⁴ Reviewed in *Berl. klin. Wchnschr.*, 1907, 44, p. 233.

⁵ *Deutsch. Arch. f. klin. Med.*, 1899, 63, p. 130.

⁶ *Ibid.*, 1901, 69, p. 47.

⁷ Reviewed in *Berl. klin. Wchnschr.*, 1907, 44, p. 233.

⁸ *Op. cit.*

of pulmonary tuberculosis, partly with the idea that it might prove a good anodyne, checking the cough and relieving the pain, and partly because he thought that a portion of the dye might be excreted in the lungs and there exert its bactericidal power, killing the tubercle bacilli within the lung tubercle. He found that the sputum of these patients always contained the dye in the form of crystals or small granules, but it was never sterile. This is the earliest report found by me of the treatment of tuberculosis with methylene blue, but it has been used by others both in the treatment of pulmonary tuberculosis and as a local application in tuberculous pharyngitis, in tuberculous glands, and in irrigating tuberculous sinuses and cavities. During the last year, the use of methylene blue in the treatment of tuberculosis has been revived by a group of German laboratory workers and clinicians. The leader, Professor Gräfin v. Linden, states¹ that living tubercle bacilli are readily stained by methylene blue (either chlorid or iodid), but are not killed by the dye unless it has been reduced by iron hydroxid. They fail to grow after 24 hours' exposure to the iron-reduced dye. She also states that tuberculous guinea-pigs treated with the dye show in the lungs grayish nodules which become blue after exposure to the air, and that these stained nodules contain blue-stained tubercle bacilli. She also finds blue-stained bacilli in the caseous substance of tuberculous lymph glands. In her first report, six guinea-pigs were treated with methylene blue. The treatments began eight days after inoculation. The dose used was 0.2 to 0.3 c.c. of a 0.1 per cent solution, the dye being injected subcutaneously and the dose repeated every day at first and later not so often. Medically pure methylene blue hydrochlorid was used at first and later the hydriodid was used instead. She says that the treatment caused healing of the initial sinus and abscess, increase of weight, lowering of fever, and prolongation of life, the controls dying in 15 to 18 weeks and the treated animals in 28 to 42 weeks. She claims that 50 per cent of the animals died from some other cause than tuberculosis and that one guinea-pig was perfectly cured and sterile, as shown by animal inoculation. All cases showed a tendency to healing as indicated by a more fibrous rather than caseous form of the disease. In her second series, the iodid of methylene blue was used instead of the chlorid, with results not essentially different from those attained in the first experiment. No other case of "perfect healing and sterilization" was reported. These animals were inoculated with 0.5 mg. of a two-months-old culture of human tubercle bacilli of an estimated virulence of 15 weeks. These small doses of cultures of low virulence were used expressly to hinder too rapid development of the disease and to allow more time for the dye to take effect. Slight as were her results, they were sufficient to encourage the use of the dye in the therapeutics of tuberculosis. At Finkler's and v. Linden's suggestion, Strauss² and Meissen³ used the dye both with and without copper in a number of patients suffering with pulmonary tuberculosis and also in a number with external tuberculosis. Meissen's report on his cases of pulmonary tuberculosis were not especially encouraging, but Strauss, who usually employed the methylene blue combined with some copper compound, claims that it caused diminution of pain and healing of fistulas and abscesses. Selter⁴ makes a controversial report, claiming that he worked with Finkler and also with v. Linden and received no credit for his part in the work. He also claims that v. Linden's results, as he saw them, and also the results obtained in his own experiments were not decisive and did not deserve so positive a report and recommendation as had been given by v. Linden.

¹ *Beitr. z. Klin. d. Tuberk.*, 1912, 23, p. 201; also *München. med. Wchnschr.*, 1912, 59, p. 2560.

² *Beitr. z. Klin. d. Tuberk.*, 1912, 23, p. 223.

³ *Ibid.*, p. 215.

⁴ *Ibid.*, 1912, 24, p. 261.

My own experiments with methylene blue had begun before the appearance of the German reports and have continued up to the present time. The first experiments were intended only to test the power of the dye to penetrate the tubercle and were reported, at least in part, in my preliminary communication. The first essential in the use of a drug or dye as a chemotherapeutic agent in tuberculosis is that it shall be able to penetrate the tubercle; better, that it shall be specific for the tubercle and be retained in it, and pile up there so as to attain a cumulative influence. Six guinea-pigs suffering with advanced tuberculosis were given subcutaneous injections of the dye, four receiving medicinally pure methylene blue (Merck) and two, Ehrlich's rectified methylene blue. The dye was used in 0.25 per cent and 1 per cent water solutions and 1-2 c.c. of the solution injected subcutaneously, one to six injections being given at intervals of two or three days. In all cases there was much infiltration and sloughing of the subcutaneous tissues and necrosis of the skin. In most cases at autopsy the tissues appeared unstained at first, but after exposure to the air and, better, after exposure to 10 per cent ammonium molybdate, the tubercles of the lungs and liver and spleen, and especially of the lungs, became blue. The normal tissue usually remained unstained, tho sometimes there was a general diffusion of the stain. In no case was I able to find tubercle bacilli stained blue, altho at times blue-stained granules were seen which could not be identified as intrabacillary granules. The caseous centers of tuberculous lymph glands generally, not always, remained unstained or less deeply stained than the periphery of the gland, and stained bacilli were never found in them. In this respect, I was less successful than v. Linden, who finds blue-stained bacilli both in the lung tubercles and in the caseous centers of glands. As a rule in my animals, the blue was present as a diffuse stain of the tubercles; still, many times, stained granules and sometimes stained nuclei were seen. Tho no quantitative estimate was made, there seemed to be no heaping-up of the dye in the tubercles, since they appeared as deeply stained in the animals that died after a single injection of 1 c.c. of a 1 per cent solution as in those that received six injections aggregating 5 c.c. of a 1 per cent solution. This is probably due to the fact that the dye is quite

rapidly excreted, as shown by Elsner and Müller, Elsner recovering about 60 per cent from the urine, and feces within three days after a single injection and Müller recovering about 70 per cent. Elsner suggests that the amount not recovered either may be deposited somewhere or may be excreted in a leuko-form which cannot be reoxidized. The latter explanation seems to me the more plausible; at least, if it is deposited, it seems to be deposited in a form that cannot be reoxidized after three or four days, since animals dying more than three or four days after the last injection rarely showed any blue in the tissues even after exposure to the air and to 10 per cent ammonium molybdate. Two guinea-pigs with advanced tuberculosis were then fed cake pills containing each about 4 mg. of medicinally pure methylene blue. One of the pigs died after only two feedings, while the other received 18 feedings of 25 pills, or 100 mg. of methylene blue. At autopsy both showed tubercles which became blue after oxidation, very much as did the animals that had been injected, and without any of the unpleasant ulcerations which had been so dangerous a feature in the injected animals. The pills were made by mixing cup cake, water, a little olive oil, and methylene blue, dividing the mass into parts as nearly equal as possible, rolling each portion between the hands into a pill, and then drying. The method resembles that recommended by Ehrlich¹ for the administration of parafuchsin and other dyes. The pills were readily eaten by the guinea-pigs and it was noted as curious that they became especially fond of the pills and eager for them as the tuberculous process advanced, so that one could predict with a good deal of certainty the approaching death of the animal from the eagerness with which it ate the pills and begged for more.

BACTERICIDAL POWER OF METHYLENE BLUE.

Having determined that a dye or drug will penetrate a tubercle, the next question of importance is: Will it enter and destroy the tubercle bacillus? With a dye, the question of penetration of the organism is easily determined by the sharpness with which the individual bacilli are stained by a given strength and in a given time. The difficulty of staining the tubercle bacillus is too well

¹ *Op. cit.*

known to need comment. Miss Sherman¹ in this laboratory has shown conclusively that the ordinary fat-soluble dyes are very poor stains for the bacilli, tho they often stain masses of culture readily and sharply. In my preliminary report, I showed that only a few of the dyes studied by me were really good stains of the individual organisms, tho most of them stained masses of organisms very well. The different types of methylene blue which I had at that time used all had the power, even in 1 per cent water solutions, of staining the individual bacilli quickly and sharply. This fact is also noted by v. Linden and by Sherman. In my earlier communication it was shown also that even very dilute solutions of the dye added to culture media had power to inhibit the growth of the tubercle bacilli, and v. Linden says that 24 hours' exposure to the reduced dye kills the organisms so that they will not grow when planted on suitable media.

In this set of experiments, the method used by me for testing the bactericidal action of the dyes was as follows: A very dilute filtered suspension of virulent human tubercle bacilli in normal salt solution is made, such that there is the merest trace of opalescence in the filtrate. One drop of this suspension is mixed with 5 c.c. of sterile 1 per cent solution of the dye to be used and left, usually at incubator temperature, for 24 hours. One drop of the mixed dye and suspension is then diluted with 5 c.c. of normal salt solution and injected subcutaneously into a guinea-pig. Microscopic examination has shown that in this method of procedure we have very few clumps of bacilli, so that each individual bacillus is surrounded by the dye and may be easily penetrated by it and killed if it has any bactericidal power. In all cases examined in which any of the methylene blues were used, the organisms were found to be well stained.

Fourteen guinea-pigs were inoculated with tubercle bacilli which had been exposed for 24 hours to 1 per cent solutions of the different preparations of methylene blue, and Table 1 gives very briefly the results. As most of these animals were used for therapeutic experiments with different dyes after the local tubercle had developed, and as the treatment may in some cases have modified the results, the treatment used is indicated in column 4 of the table.

¹ *Jour. Infect. Dis.*, 1913, 12, p. 249.

Of this series, No. 2 was especially interesting. The animal remained well, its nutrition was unusually good, and it bore several litters of healthy young. Finally, 9 months after inoculation, it was

TABLE 1.

Dye Used	Time before Local Tubercle Developed	Time before Death	Treatment Used, If Any	Postmortem Findings
1. Ehrlich's rectified methylene blue....	None developed	9 months (killed)	None	No tubercles found. Animal normal
2. Medically pure methylene blue (Merck's).....	None developed	9 months (killed)	None	No local or general tuberculosis. Three encapsulated and apparently sterile nodules and some adhesions in abdomen
3. Medically pure methylene blue (Merck's).....	25 days	6 months	Methylene blue	Caseous gland in groin. Enlarged and necrotic spleen. No other involvement
4. Ehrlich's rectified methylene blue....	2.5 months	4 months	Silver trypan blue	Slight local and marked general tuberculosis
5. Ehrlich's rectified methylene blue....	2.5 months	3.5 months	Silver trypan blue	Small local tubercle. Liver and spleen necrotic. No other involvement
6. Ehrlich's rectified methylene blue....	46 days	75 days	Methylene blue (feeding)	Local and general tuberculosis
7. Medically pure methylene blue....	45 days, but disappeared	99 days	Selenium blue	No local tubercle. Spleen and liver large and necrotic. Few small tubercles in lung
8. Medically pure methylene blue....	45 days and developed slowly	93 days (killed)	Methylene blue (feeding)	Small local tubercle. Small tubercles in omentum. Very little other involvement
9. Medically pure methylene blue....	33 days and developed slowly	98 days	Lithium carmine	Very small local tubercle. Lungs filled with tubercles. None in liver and spleen
10. Methylene blue iodid	18 days	4 months	Congo red	Slight local and marked general tuberculosis
11. Methylene blue iodid	18 days	5 months	Congo red	Slight local and marked general tuberculosis
12. Methylene blue iodid	18 days	2.5 months	Congo red	Local and general tuberculosis
13. New methylene blue N.....	25 days	6 months	Methylene blue	Local and general tuberculosis
14. New methylene blue GG.....	32 days	133 days (killed)	Methylene blue	No local tubercle. Spleen soft, but no tubercles. Few necrotic areas in liver and lung

killed. Autopsy showed rolls of adipose tissue everywhere. No sign of malnutrition. None of the superficial glands were enlarged and all organs appeared normal, altho there were a few old adhesions around the spleen. Three sharply circumscribed, thick-walled

nodules filled with soft, cheesy, puruloid material were, however, found in the abdomen, one just under the anterior abdominal wall and the other two in the great omentum near the spleen. Smears from the interior substance of these nodules showed no acidfast bacilli and indeed no bacteria of any kind. Unfortunately no animal inoculations were made, but the findings seem to indicate that in this case some of the tubercle bacilli survived the treatment with methylene blue and remained capable of growth, but with virulence so weakened that no general infection resulted; the tuberculous process showed a marked tendency to healing and encapsulation and the organisms finally died out.

From Table 1, it may be seen that methylene blue in none of the preparations used can be depended on as a bactericidal agent. In one case all the organisms seem to have been killed, and in another, much attenuated. In all the tests, except those with methylene blue iodid, the local tubercle was slow in developing and generally remained small, while in some cases, either no local tubercle ever developed or one developed and later disappeared. It is difficult to draw conclusions from the very irregular time of death, since the animals, after the discovery of a local tubercle, were used for testing the therapeutic action of different dyes, some of which may have hastened and some delayed the exitus of the animal. It is to be noted that a slight or no local involvement is the rule and in many of the cases the general extension was so slight that one could scarcely ascribe the cause of death to the tuberculous involvement and must believe that some other cause intervened. Only one of the 14 animals, however, was entirely free from infection.

THERAPEUTIC EXPERIMENTS WITH METHYLENE BLUE.

In beginning my therapeutic experiments with methylene blue, it seemed to me necessary, because of the ulceration and sloughing caused by even the purest medicinal preparations of methylene blue which were employed in my earlier experiments, to use the dye in very dilute form and in very small doses. The first series of guinea-pigs subjected to treatment with the dye had been inoculated in experiments undertaken to test the bactericidal action of various dyes. The treatment was therefore begun relatively late in the

disease, since it was desired to find a palpable local tubercle before interrupting the disinfectant experiment. In all cases where no preparation is mentioned, Merck's medicinally pure methylene blue is the preparation used.

1. Treatment begun 35 days after inoculation. Subcutaneous injections of 0.5 to 1.0 c.c. of a 0.1 per cent solution were given at intervals of 2-3 days. Forty-eight injections were given, totaling 32 mg. of the dye. Death, 144 days after inoculation. Local glands enlarged and caseous. Spleen large and necrotic. Lungs and liver not involved. Tubercles deep blue.

2. Treatment begun 35 days after inoculation. Fifty-one injections given, totaling 54 mg. of methylene blue. Died 152 days after inoculation. A few enlarged and caseous glands in groin. Spleen, liver, and lungs contained a few miliary tubercles. General tuberculosis not advanced. Acute peritonitis, pericarditis, and pleurisy existed and were probably the cause of death.

3. Treatment begun 35 days after inoculation. Killed on one hundred and thirty-third day, after 45 injections containing 39 mg. of the dye had been given. A foul-smelling, necrotic ulcer in abdominal wall from action of dye. No local tubercles. Spleen large and soft, but showing no tubercles or necrotic areas. A very few small necrotic areas in liver and lungs. Tuberculous process very slight. (These three animals had been inoculated with tubercle bacilli which had been exposed for 24 hours to methylene blue. Hence the low virulence of the infection may be due to this fact rather than to the after treatment with the dye.)

4. This pig was inoculated with a culture which had been exposed for 24 hours to 1 per cent neutral red. No definite tubercles were found until the forty-seventh day after inoculation, but a curvature of the spine in the cervical region and partial paralysis developed on the fortieth day. Treatment with methylene blue was begun on the forty-seventh day, at which time the pig was so weak and ill and emaciated that it was thought to be near death. However, it improved rapidly after the methylene blue treatment was begun; it gained in weight and general condition; the curvature and paralysis both improved, altho the head always tended to turn to one side. About 50 injections were given, making approximately 53 mg. of the dye. The animal died 186 days after inoculation, its nutrition still being excellent. The sloughing of the skin and subcutaneous tissues had become so bad that no injections were given during the last two weeks of life. One gland in groin was enlarged, but hard, not caseous. The liver was pale and hard and showed a few greenish areas of necrosis. The spleen was large and soft and imbedded in a mass of partly coagulated blood. The lungs appeared normal. Death seemed to have been caused by rupture of the spleen and hemorrhage.

5. Trypan red was the disinfectant used in this case and a local tubercle was found 19 days after inoculation. Methylene blue treatment was begun 35 days after inoculation. Forty-three injections, making 42 mg. of methylene blue, were given. The pig died 131 days after inoculation, showing a large group of caseous glands in the groin, spleen and liver large and necrotic, and lungs filled with larger and smaller tubercles, many of which contained cavities.

6. This pig was inoculated with human tubercle bacilli which had been exposed for 24 hours to 1 per cent bluish eosin. Treatment with methylene blue was begun 35

days later. Forty-three injections were given, totaling 42 mg. of methylene blue. Death occurred 130 days after inoculation. Several glands in the groin were enlarged and caseous. The liver and spleen were large and necrotic. The lungs were filled with tubercles, many containing cavities.

7. This pig developed paralysis of the leg of the infected side after 40 days, but no local tubercle until 47 days after inoculation. Methylene blue was begun on the forty-eighth day and continued until death, which occurred 105 days after inoculation. Glands in groin were large and hard, but not caseous. Liver and spleen were large and necrotic. Lungs were filled with tubercles, some large, some small, the larger ones generally containing cavities.

The next series of animals were inoculated with a large dose of virulent culture of human tubercle bacilli. Treatment was begun two weeks after inoculation, the same method of treatment and the same doses being used as in Series 1. The four untreated controls had all developed local tubercles by the twenty-first day and died of local and generalized tuberculosis in 58, 126, 138, and 231 days.

1. Forty-two injections were given, making a total of 30 mg. of methylene blue. Death occurred 112 days after inoculation. There had been much necrosis and sloughing of skin and subcutaneous tissue. One gland in groin was enlarged and caseous. Liver hard and filled with tubercles and necrotic areas. Spleen moderately enlarged and almost entirely necrotic. Lungs dotted with young tubercles. Abdomen filled with bloody fluid.

2. Forty injections, making a total of 27 mg. of the dye, were given. Died on the one hundred and fifth day after inoculation. Caseous gland in groin; liver and spleen large and necrotic; lungs filled with tubercles. Pleural cavity filled with a serous exudate.

3. Forty-two injections, making a total of 33 mg. of methylene blue. Death on one hundred and twelfth day. Large group of caseous glands in groin. Liver very sclerotic with necrotic border. Spleen large and necrotic. Lungs filled with tubercles. Abdomen filled with clear fluid.

4. Thirty-six injections, making in all 24 mg. of methylene blue. Death 89 days after infection. Large caseous gland in groin. Liver not involved. Spleen slightly enlarged and shows a few miliary tubercles. Lungs show no tubercles. Pleural cavity filled with a thick, white, turbid, semi-solid exudate, covering lung surface and pericardium. No acidfast organisms were found in this exudate.

5. Forty-eight injections, in all 34 mg. of methylene blue. Death 134 days after infection. Two slightly enlarged, hard, not caseous glands in groin. Spleen large and filled with tubercles. Liver hard and pale. Lungs showed many tubercles. Pleural cavities filled with serous exudate.

As I had earlier found that the so-called new methylene blues penetrated the tubercle very well and also stained the tubercle bacilli and inhibited their growth, seven of the new methylene blues were used to treat as many guinea-pigs, the treatment being begun, as in the last experiment, two weeks after infection.

1. New methylene blue R. Forty-three injections were given, making a total of 47 mg. of the dye. Death occurred on the one hundred and twenty-ninth day. Slightly enlarged gland in groin and miliary tubercles in lungs, liver, and spleen.
2. New methylene blue GB. Thirty-seven injections, equaling 25 mg. of dye. Death on one hundred and sixth day. Caseous glands in groin. Liver and spleen enlarged and necrotic. Lungs free from tubercles.
3. New methylene blue N. Forty-five injections, making about 49 mg. of the dye. Death 133 days after inoculation. No local tubercle. Liver pale and hard; no tubercles. Spleen large, white, and necrotic. Lungs firm and dotted with tubercles.
4. New methylene blue NSS. Thirty-six injections, or 36 mg. of the dye. Died 100 days after infection. Caseous glands in groin. Liver and spleen and all abdominal organs appear normal. Lungs show no tubercles. Pleural cavity filled with a thick, yellowish-white, semi-solid exudate. No acidfast organisms in smears from this exudate, but many cocci and short, thick bacilli.
5. New methylene blue GG. Forty-five injections, making in all 44 mg. of the dye. Injections stopped some weeks before death, because of the bad condition of the animal. Died 140 days after infection. Slightly enlarged, but not caseous glands in groin. Abdominal and thoracic cavities filled with thick, turbid exudate. Lungs and liver contained a few small tubercles, but death was evidently due to acute pyogenic infection causing peritonitis and pleurisy.
6. New methylene blue NX. Much infiltration and necrosis of skin. Thirty-one injections, containing 34 mg. of the dye. Died 86 days after infection. Local gland slightly enlarged and hard, but not caseous. Liver and spleen large and very necrotic. Large tubercles in lungs, many containing cavities.
7. New methylene blue 3R. Thirty-five injections, making 39 mg. of the dye. Died 99 days after infection. Glands in groin large and caseous. Spleen and liver large and necrotic. Lungs showed a few tubercles.

Since v. Linden reports that she finds the iodid of methylene blue to have certain advantages over the chlorid, among them being that it is less irritant, I used some pure methylene blue iodid on a series of animals. This dye was 30.94 per cent iodin, and since iodin was found by Wells and Hedenburg¹ to penetrate the tubercle especially well, it was thought possible that this factor might cause the dye to penetrate the tubercle better and at the same time increase its bactericidal and therapeutic power. So far as its bactericidal power is concerned, however, it has proved disappointing, since, as shown in Table 1, the animals inoculated with tubercle bacilli which had been exposed to this salt of methylene blue all developed local tubercles in 18 days, much earlier than was the case with any of the other methylene blues, and death occurred in from two and one-half to five months in all cases, with marked general tuberculosis. The guinea-pigs used for treatment with this salt of

¹ *Jour. Infect. Dis.*, 1912, 11, p. 349.

the dye had been inoculated subcutaneously with a large dose of virulent human tubercle bacilli. Treatment was begun three weeks after inoculation, 0.1 per cent solution being employed and from 0.5 to 1.0 or more cubic centimeters being used at each dose.

1. Forty-one injections were given, making 33 mg. of the dye or 10.21 mg. of iodine. Death 149 days after inoculation. Large caseous gland in groin. Spleen and liver enlarged and showing necrotic areas. Lungs exhibit a few small tubercles. Some turbid fluid in abdomen, and pleural cavity filled with thick, semi-solid, yellowish-white exudate.

2. Twenty-four injections given in 53 days, totaling about 13 mg. of the dye or 4.02 mg. of iodine. Death 77 days after infection. Local glands slightly enlarged; not caseous. Spleen and liver enlarged and filled with tubercles and necrotic areas. Lungs not involved.

3. Twenty-five injections in 55 days, totaling 15 mg. of dye or 4.6 mg. of iodine. Died 79 days after inoculation. Two enlarged caseous glands in groin. Spleen and liver greatly enlarged and filled with tubercles and small necrotic areas. Lungs dotted with miliary tubercles.

4. Thirty-five dye injections made, totaling 20 mg. of dye or 6.18 mg. of iodine. Death 108 days after inoculation. Glands in groin enlarged and caseous. Liver enlarged, white, and hard, with a few necrotic areas. Spleen almost entirely necrotic. Lungs full of tubercles. Pleural cavity filled with serous exudate.

5. Forty-four injections given, totaling about 24 mg. of dye or 6.8 mg. of iodine. Animal died 140 days after infection. Local glands only slightly enlarged, not caseous. Spleen and liver very necrotic. Lungs dotted with small, hard, white nodules.

All tubercles turned blue on exposure to air or molybdate, with methylene blue iodid as well as with the chlorid.

After several experiments, earlier described, showing that cake pills of methylene blue could be fed to tuberculous guinea-pigs and that the dye administered in this way penetrated the tubercle, it was decided to try treating some tuberculous pigs with methylene blue by feeding the pills, since the subcutaneous injections, no matter how pure the preparation, nor how carefully graded the dose, nor how aseptically the injection is made, always in time and after many treatments cause serious ulcerations, which greatly increase the danger of secondary infections.

Each of six guinea-pigs first received a subcutaneous injection of 0.5 c.c. of a 0.3 per cent solution of medicinally pure methylene blue and each day afterward was fed a pill containing approximately 4 mg. of the dye. The strength of the pills was gradually increased up to 8 mg. per pill, or dose. Eight days after the first dye injection, each pig received a subcutaneous injection of 0.3 c.c.

of a dilute, filtered suspension of a three-weeks-old culture of human tubercle bacilli. Untreated controls died 75, 69, 80, 169, and 194 days after inoculation, all showing tuberculous lungs, generally necrotic spleens and livers, but none showing caseous glands at the site of inoculation.

1. Pig died 74 days after inoculation. Had received approximately 328 mg. of the dye by mouth. Local glands enlarged and caseous. Liver and spleen filled with tubercles and necrotic areas. Lungs packed with miliary tubercles and pleural cavities filled with serous exudate. Left adrenal showed a tubercle on anterior surface.

2. Death 80 days after inoculation after receiving about 360 mg. of dye by mouth. No local tubercle. Liver pale, yellowish in color, with a few deep-yellow areas. Spleen large, soft, deep red. No tubercles. Lungs nearly normal. Cause of death not determined, but not tuberculosis.

3. Death 101 days after infection. Spleen larger than normal and dotted with tubercles. Peribronchial glands large and lungs dotted with tubercles. Feeding was stopped some weeks before death.

4. Death 80 days after infection. Several enlarged and caseous glands in each axilla and in each groin. Liver and spleen enlarged and necrotic. Spleen completely necrotic—pale yellow—no normal spleen tissue. Lungs filled with tubercles.

5. Death 99 days after infection. Several enlarged and caseous glands in axilla. Spleen and liver enlarged and a mass of tubercles. Lungs only slightly involved.

6. Death 113 days after inoculation. Spleen one large necrotic mass. Liver full of necrotic areas. Lungs filled with miliary tubercles; peribronchial and retroperitoneal lymph glands enlarged.

In order to determine whether a single large injection of methylene blue given very soon after the infection of the animal would prevent the development of tuberculosis, four guinea-pigs were given, 24 hours after inoculation, 1 c.c. of a 0.5 per cent solution of methylene blue, two receiving it by subcutaneous injection at site of infection, and the other two intraperitoneally. One died on the eighth day, showing no sign of development of tuberculosis. The other three had developed local nodules by the twelfth day after infection and all developed a marked general tuberculosis.

Sellei¹ in 1912 published a report on the influence of dyes combined with poisons and therapeutic agents. Among other things, he states that, while the addition of methylene blue increases the toxic action of most copper salts, copper chlorid, which is the most toxic of these salts, becomes much less poisonous on the addition of methylene blue. One cubic centimeter of 1 per cent CuCl_2 to each 100 gm. body weight of animal injected subcutaneously kills guinea-

¹ *Biochem. Ztschr.*, 1913, 49, p. 466.

pigs in five to eight hours. Mixed with 0.3 c.c. of 1 per cent methylene blue, the animals live 12 to 16 days or longer. A similar diminution of toxic action of ferrous sulfate was obtained by mixing methylene blue with it, altho ferric chlorid with methylene blue kills much more quickly than the salt without the dye.

As the effect of numerous copper compounds on tuberculosis has been tested in this laboratory by Dr. Corper,¹ and as none of those tried has been found effectual, and as v. Linden and her associates have claimed good results from a combination of methylene blue with copper compounds, I inoculated eight guinea-pigs with human tubercle bacilli and 24 hours later gave four of them a subcutaneous injection of 2 c.c. of copper chlorid and methylene blue (25 c.c. of 1 per cent CuCl_2 +3 c.c. of 1 per cent methylene blue). Twenty-four hours after infection the other four received a subcutaneous injection of 2 c.c. of ferrous sulphate+methylene blue (20 c.c. of 1 per cent FeSO_4 +1 c.c. of 1 per cent methylene blue). Two of the pigs treated with CuCl_2 died within the first week showing no sign of tuberculosis. In all four there was very great infiltration and sloughing, so that no further injections were given and palpation would not reveal the presence of a tuberculous nodule. Two died early but the other two developed local and general tuberculosis. Of the four treated with FeSO_4 and methylene blue, one died on the eighth day with no sign of tuberculosis; another died 25 days after infection with two enlarged and partly caseous glands in groin. The other two exhibited local nodules on the twelfth day, which were discharging a caseous substance on the twenty-fifth day and died, one on the thirty-seventh and the other on the fifty-fifth day with marked generalized tuberculosis. Only one injection was given on account of the infiltration and ulceration following the first. These results do not suggest that a combination of these metallic salts with methylene blue can be used effectually or safely in the treatment of tuberculosis.

The results of treatment of experimental tuberculosis as shown by these tests are hardly as encouraging as one is led to expect from v. Linden's reports. She states that methylene blue penetrates the tubercle and stains the bacilli within the tubercle and even in

¹ *Jour. Am. Med. Assn.*, 1913, 60, p. 887.

the caseous centers of tuberculous lymph glands. This may easily be true, as it certainly penetrates the younger epithelioid tubercles, tho not often the centers of old, caseous tuberculous lymph glands. I, however, have never succeeded in finding blue-stained organisms in the tubercles of any of my animals, altho it would seem that, if they were as uniformly stained as is suggested by v. Linden's report, they might have been found in some of my treated animals. She also says that treatment with methylene blue chlorid or iodid causes healing of the initial sinus and abscess, increase of weight, lowering of fever, and prolongation of life, while 50 per cent of her six animals died from some cause other than the tuberculous infection, and one was entirely healed and sterile, as shown by animal inoculations. An absolute comparison of my results with hers is perhaps not fair, as she used a culture of low virulence, while mine was always a young, highly virulent culture. It is true that the guinea-pig is so susceptible to tuberculosis that therapeutic experiments on guinea-pig tuberculosis are more difficult and discouraging than on other animals or on man, but for that very reason we may infer that a therapeutic agent which heals the disease in the guinea-pig will be much more efficacious in man; but not if we facilitate the cure by using non-virulent cultures or those of low virulence. In my six feeding experiments, the initial sinus and abscess did discharge and dry up and heal either entirely or nearly. In one case a deep nodule later broke through the old, partly healed fistulous tract and it was discharging at the time of death. This might confirm one of v. Linden's contentions, were it not for the fact that in the five untreated controls of the same series the same thing occurred and in these no deep nodules formed, so that at death there were no local tubercles and the initial sinus was completely healed. Hence, this can hardly be ascribed to the methylene blue. In none of the other series was a healing of the local tubercle noted except in exceptional cases. The progressive weight and fever curve have not been systematically recorded in my cases, so that I cannot compare mine with hers in this respect. As to prolongation of life, Table 2 will epitomize my experience. Since it is fair to compare treated animals only with controls of the same series in which the same culture and doses were used, the results

have been tabulated in series. The first series had no definite controls and other factors entered in to modify results, as explained in the description of this series, but results will be tabulated so far as length of life is concerned.

TABLE 2.

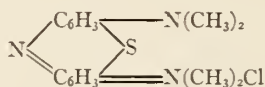
SERIES 1	SERIES 2				SERIES 3	
Medicinal Methylene Blue Days	Control Days	Medicinal Methylene Blue Days	Methylene Blue Iodid Days	New Meth- ylene Blues Days	Control Days	Medicinal Methylene Blue Days
144	58	112	149	129 (R)	75	74
152	126	105	77	106 (GB)	69	80
133	138	112	79	133 (N)	80	101
186	231	89	108	100 (NSS)	169	80
131	134	140	140 (GG)	194	99
130	86 (NX)	113
106	99 (3R)
Average 140 $\frac{2}{7}$	138 $\frac{1}{2}$	110 $\frac{2}{3}$	110 $\frac{2}{3}$	113 $\frac{2}{7}$	117 $\frac{2}{3}$	91 $\frac{1}{2}$

A study of this table shows first that the duration of life, even in the controls of a single series, has so wide a range of variation that the conclusion is never justified that the process is less severe or is being favorably influenced by a given treatment simply because life is longer. Second, if we do attempt to compare averages, we find that the average duration of life of the treated animals is slightly less than that of the untreated of the same series, instead of being much greater, as v. Linden found it. As to her statement that three of her six animals died from some intercurrent affection instead of tuberculosis, altho they had that disease also, it is not at all unusual, even in guinea-pigs infected with tuberculosis, for death to be due to some cause other than the experimental disease in question. Guinea-pigs are susceptible to nearly all infections as well as to tuberculosis, and the tuberculous infection may even predispose them to some other fatal infection. For instance, 4 of the 7 pigs of my first series, 4 of the 17 of the second series, and 1 of the 6 in the third series, thus 9 of the 30 treated pigs, died, not from tuberculosis, but from some other condition, and in most of these the tuberculous involvement was very slight and not sufficient to be regarded as the cause of death. In many cases also, as in v. Linden's, such tubercles as were present, or at least some of the

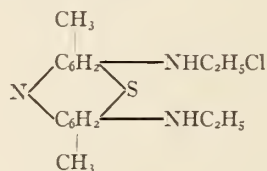
tubercles present, were hard and fibrous rather than caseous and might perhaps be regarded as in process of healing.

While there may have been some improvement in a few of the cases therefore, the results have not been so perfect or so uniform that methylene blue should be advised as a cure for tuberculosis, tho it may possibly have some palliative effect. The fact, however, that the dye seems to have some bactericidal power, either killing or markedly modifying the virulence of the human tubercle bacilli in nearly every case, makes it seem possible that this dye may serve as a starting-point for the development of a dye or chemical substance which may have a stronger and more specific influence on the tuberculous process than has methylene blue itself.

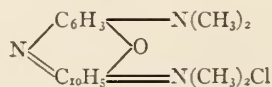
As reported, I have already used the iodid of methylene blue in place of the chlorid and have also used seven of the so-called new methylene blues which are but modifications of methylene blue. Methylene blue is a quinone imide thiazin dye, having the structural formula:



New methylene blues N and NSS are also quinone imide thiazin dyes, having the structural formula:



These dyes are no more efficient than methylene blue itself and are quite as irritating to the skin. New methylene blue GG is an oxazin instead of a thiazin dye and also replaces one C_6H_3 group by C_{10}H_5 . The formula is as follows:



This dye penetrated the tubercle well, was fairly well borne, and the animal died of an acute peritonitis 140 days after inoculation, the tuberculous process being very slight. This dye and also

Capri blue, another oxazin derivative of methylene blue, will therefore be tested further. As to the other new methylene blues, I have not been able to find the formulae and most of them appear to be mixtures rather than pure dyes. They also are not promising as therapeutic agents.

In considering the question of feasible modifications of the methylene blue formula which could be made in the laboratory, the sulfur atom seemed naturally the first point of attack. Gosio¹ in 1905 suggested the use of sodium and potassium salts of selenium and tellurium as indicators of the life of bacterial cells, and Belfanti² in 1912 confirmed his results and extended his observations to the study of the tubercle bacilli. Both noted that, while 1 to 50,000 or 1 to 100,000 could be used with safety and would penetrate the bacteria and be reduced, a percentage higher than 1 in 10,000 was likely to kill the bacteria or at least inhibit their growth. Belfanti states that the tubercle bacilli, when actively living, become saturated with these salts and reduce them, and suggests that these salts of selenium and tellurium have a pronounced bacteriotropic power for the tubercle bacillus and could be used as the point of origin for new medicamentous preparations in the sense of Ehrlich. It was with this suggestion in mind that I decided to attempt to modify the methylene blue molecule by replacing the sulfur atom by an atom of selenium and also by one of tellurium, hoping in this way to combine the penetrating and reducing and bactericidal power of the selenium and tellurium with the milder bactericidal power and relative innocuousness of the methylene blue. The chemical processes were carried on for me by Dr. Walter Fraenkel; in the following I am giving his figures, and in the main his description of the method used by him. The same apparatus was used for making both dyes and is represented in Figs. 1 and 2.

A, Fig. 1, is filled with zinc and dilute hydrochloric acid. The hydrogen gas generated is dried in *B* with concentrated sulfuric acid. In *C* we place phosphorus selenide, made by fusing 11 parts of red phosphorus with 66 parts of finely pulverized selenium. Two grams of dimethylparaphenyldiaminechlorhydrate are dissolved in 200 c.c. of boiled, still hot water to which has been added 80 c.c. of concentrated hydrochloric acid. This solution is placed in flasks *D* and *E*, and *F* and *G* are filled half full of dilute permanganate solution, in order to avoid the escape of that portion of the

¹ *Ztschr. f. Hyg.*, 1905, 51, p. 65.

² *Ztschr. f. Chemother.*, 1912, 1, p. 113.

hydrogen selenide which has not entered into the reaction and the breathing of which is very dangerous. After a current of hydrogen gas has been passed through the apparatus to remove all the air, about 150 c.c. of sodium hydroxid is pressed with the help of a rubber bulb through the dropping funnel into the Erlenmeyer flask *C* and then allowed to stand some hours, with frequent shaking and slow passage of hydrogen gas, until the sodium hydroxid is colored dark brown. After the hydrogen stream has been cut down to a few bubbles to the minute, concentrated sulfuric acid is added drop by drop to the sodium hydroxid. The hydrogen selenide thus formed is allowed to pass through the apparatus for about 15 minutes. Then 60 c.c. of a 10 per cent solution of iron chlorid is added to *E* through the dropping funnel. After this has been well shaken, the rubber connections between *D* and *E* and between *E* and *F* are removed and *D* is connected with *F*. The fluid in *E* has now become green and is tested in a reagent glass to see whether it becomes darker green on the addition of more iron chlorid. If it does, iron chlorid is added gradually until tests do not become

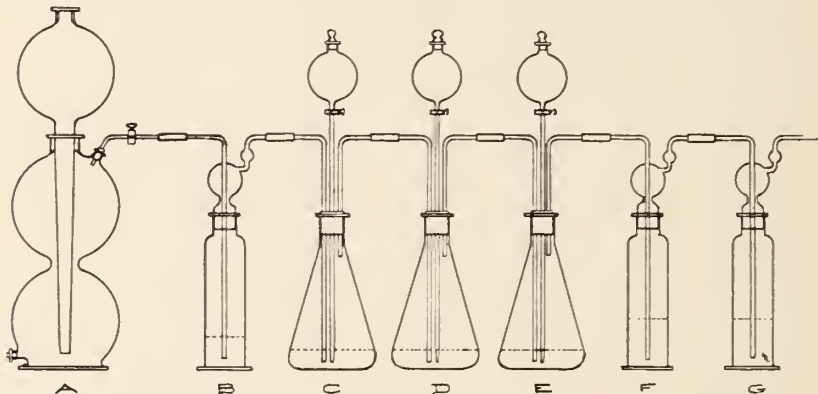
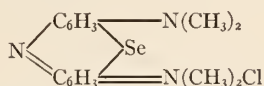


FIG. 1.—A, Kipp's apparatus; B, F, and G are wash bottles; C, D, and E are 500 c.c. Erlenmeyer flasks.

greener. The same is done with Erlenmeyer flask *D*. After it has been filtered with the aid of a suction pump, the fluid is placed in a shaking funnel and shaken with 100 c.c. of melted phenol. If, after standing, the two layers do not clearly separate from each other, a little concentrated hydrochloric acid is added. After the phenol is removed, the operation is repeated with new phenol and the two solutions mixed. This phenol solution is shaken four times with water, while each time after shaking a larger amount of hydrochloric acid is added to prevent the dye being taken up by the water. To the phenol solution are added 100 c.c. of ether and 100 c.c. of dilute hydrochloric acid and the mixture is strongly shaken. The dye then goes over into the water and the phenol-ether solution remains almost colorless. The dye solution is now shaken out several times with ether and finally allowed to stand over ether for 24 hours. After the separation of the ether and filtering, a part of the solution is placed in a small—not over 200 c.c.—distilling flask and distilled at a temperature of 30° to 40° to remove the water and hydrochloric acid. In order to have the boiling uniform and to prevent the entrance of air, carbon dioxide is conducted into the flask through a capillary tube, as shown in Fig. 2. After distillation, the lower part of the flask, containing the dye, is cut off, dried, and weighed; the dye is washed out with water and the flask dried and weighed. The difference gives us the amount of dye in the solution, the volume of which we measure.

This new dye is a blue dye, very soluble in water. It is not as stable as methylene blue; indeed it cannot be evaporated to complete dryness without breaking down. It is therefore necessary to keep the dye in water solution and even then the dye gradually breaks down, as shown by a precipitate in the bottom of the bottle. The formula for the dye is:



in which 21.6 per cent is selenium. The chemical reactions in the process of making this dye are the same as in the original method of making methylene blue used by Caro in 1876, hydrogen selenide being used instead of hydrogen sulfide.

In making the tellurium blue, flask C is carefully dried and in it is placed aluminium telluride, made by fusing four parts of pulverized tellurium with eight parts of aluminium. After hydrogen gas has been allowed to pass through the apparatus for some time, 30 per cent phosphoric acid is added drop by drop through the dropping funnel to the aluminium telluride. In this case, the oxidation is effected by means of hydrogen peroxid instead of by iron chlorid. After the addition of the hydrogen peroxid, the fluid is filtered as rapidly as possible and 100 c.c. of phenol added. The fluid and phenol are placed in a shaking funnel and shaken as often as the solution becomes blue. By this means, the blue dye is taken up by the phenol and the action of the hydrogen peroxid is stopped. This is repeated until the solution begins to become reddish, instead of blue. Then the phenol is poured off and purified in the manner described for the selenium blue. To remove the dye from the phenol, carbon tetrachlorid is used instead of the ether. It is then shaken with concentrated hydrochloric acid. (Very frequent shaking is necessary in order to get all the dye out of the phenol.) As the dye gradually becomes colorless, the completeness of the removal of the dye is tested by adding hydrogen peroxid to a small amount of the hydrochloric acid solution. The shaking is repeated until dark-blue color no longer follows the addition of the hydrogen peroxid. The rest of the treatment is the same as that for the selenium blue. This new dye is dark blue, soluble in water, even less stable than the selenium blue, cannot be completely dried, and even in water solution soon breaks down. Its formula is:

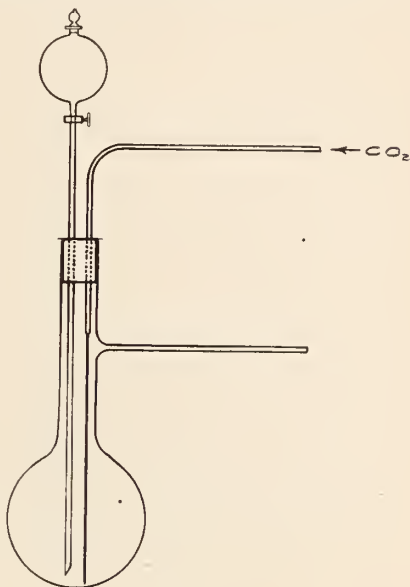
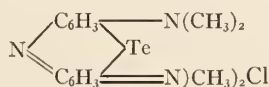


FIG. 2.



It contains 33.5 per cent of tellurium.

Both the selenium blue and the tellurium blue were, as stated before, rather unstable, especially the tellurium, and it was therefore necessary to use them as soon as possible after they were made. For this reason, the number of experiments is not so large as it might otherwise have been, altho of course more dye would have been made if the tests had been more encouraging. Both dyes were water-soluble, staining living tubercle bacilli fairly well, and fixed bacilli not so well. The stain, however, was never so clear and sharp as that obtained with pure methylene blue.

BACTERICIDAL ACTION OF SELENIUM BLUE AND TELLURIUM BLUE.

When these dyes were added to glycerin agar and human tubercle bacilli planted on the mixture, there was a more or less deep staining of the clumps which always failed to grow; in this respect, the new dyes resembled methylene blue. In the form of a very dilute, filtered suspension, human tubercle bacillus culture was also exposed for 24 hours to the action of 1 per cent solutions of these dyes, and one drop, diluted with 5 c.c. of normal salt solution, was injected subcutaneously into guinea-pigs, with the following results:

SELENIUM BLUE.

1. Large nodule found in axilla 18 days after inoculation. This soon ruptured, discharged, and nearly healed. Death 65 days after inoculation. Slight enlargement of axillary glands. Lungs, liver, and spleen show moderate involvement.

2. Eighteen days after inoculation, a discharging abscess was found at site of entrance of needle. This nearly healed. On thirtieth day, a deep nodule was found in axilla. Death about four months after inoculation. Enlarged and caseous gland in axilla. Lungs, liver, and spleen contained tubercles.

3. As in the other cases, nodule developed early before the eighteenth day and ruptured, discharged, and then healed. No glands became enlarged or caseous. Death occurred on the sixtieth day, lungs, liver, and spleen being full of miliary tubercles.

4. Again an initial abscess formed, discharged, and healed. On sixtieth day a deep nodule was found in axilla. Death on ninety-fifth day. Enlarged and caseous gland in axilla. Peribronchial and retroperitoneal glands enlarged. Liver, spleen, and lungs full of tubercles and necrotic areas.

TELLURIUM BLUE.

1. Abscess developed, discharged, and healed within the first month. On sixtieth day a small nodule was found in axilla. Pig was killed 158 days after inoculation, because it was weak and emaciated and hind parts were paralyzed. No enlarged or

caseous glands in axilla. Lungs showed a few small, translucent areas and one cavity-containing tubercle. Liver normal. Spleen showed a few small tubercles. Extent of tuberculous process hardly sufficient to account for condition.

2. Abscess ruptured and discharged early. On sixtieth day a deep nodule was discovered in axilla. Died on one hundred and thirty-third day. Lungs, liver, and spleen contained tubercles. Caseous gland in axilla.

3. Initial abscess ruptured and healed early. Death on one hundred and eighty-ninth day. No local tubercle. Lungs, liver, and spleen contained tubercles. Thorax full of serous exudate.

4. Initial abscess ruptured and healed early. Deep nodule found on sixtieth day. Death 125 days after inoculation. Enlarged and caseous gland in axilla. Peribronchial and retroperitoneal glands enlarged. Lungs, liver, and spleen contained tubercles.

A comparison of these with the controls of the same series shows no or very little bactericidal power in these dyes, as may be seen from Table 3. The average duration of life is in favor of the tellurium blue.

TABLE 3.

Dyes Used	Initial Abscess		Local Tubercle Days	Death	Postmortem Changes
Control	18 days.	Discharged and healed	31	140 days	Local and general tuberculosis
	18 days		32	4 months	" " " "
	18 days.	Discharged and healed	61	75 days	" " " "
	18 days.	Healed	31	4 months	" " " "
					" " " "
Selenium blue	18 "	"	Very late	65 days	Slight local and general tuberculosis
	18 "	"	31	4 months	Local and general tuberculosis
	18 "	"	None	60 days	No local; marked miliary tuberculosis of organs
	18 "	"	60	95 "	Local and general tuberculosis
Tellurium blue	18 "	"	60	158 "	No local and slight general tuberculosis
	18 "	"	60	133 days	Local and general tuberculosis
	18 "	"	60	189 "	" " " "
	18 "	"	60	125 "	" " " "

AVERAGE DURATION OF LIFE AFTER INOCULATION.

Controls	Selenium Blue	Tellurium Blue
112 days	100 days	151 days

THERAPEUTIC EXPERIMENTS WITH THE SELENIUM AND TELLURIUM BLUES.

Trial experiments with guinea-pigs having advanced tuberculosis showed that these dyes had about the same effect on the skin as did methylene blue and also that they penetrated the tubercle, were reduced there, and could be oxidized to a blue dye by action of 10

per cent ammonium molybdate. In other words, they behaved very much like methylene blue, but were stronger toxins and weaker dyes. On account of the effect on the skin and the very small dose that could be given subcutaneously, the method of feeding with cake pills was used in the series treated with these new dyes. As in the series fed with methylene blue the animals first received one subcutaneous injection followed by daily feedings. Eight days after the first injection each animal received a subcutaneous injection of 0.3 c.c. of a dilute, filtered suspension of human tubercle bacilli and the daily feeding of the pills was continued.

1. An abscess was found ruptured and discharging 19 days after inoculation. This healed and a deeper gland in axilla gradually enlarged. Died 68 days after inoculation. No autopsy.

2. An abscess developed by the nineteenth day after inoculation. This later ruptured, discharged, and healed. No other local tubercle developed. Death 94 days after infection. Liver normal. Spleen large and necrotic; lungs a mass of miliary tubercles.

3. A discharging abscess found on nineteenth day. This partly, but never entirely, healed. Death 81 days after infection. Slightly enlarged and slightly caseous gland in axilla. Liver pale and filled with necrotic areas. Spleen enlarged and filled with miliary tubercles. Apices of both lungs showed many advanced tubercles, while the rest of lungs had only a few small tubercles.

4. Nodule at point of inoculation on nineteenth day. This discharged and healed. Death 95 days after infection. No local tubercles. Liver and spleen large, soft, and deep red. No tubercles visible. Lower lobes of both lungs solid and filled with miliary tubercles.

5. Nineteen days after inoculation, a discharging nodule was found at point of inoculation. This crusted over and partly healed, but broke open again to emit discharge from deeper tuberculous glands. Death on ninety-sixth day. Both axillae contained numerous large, caseous, tuberculous glands. Liver, spleen, and lungs filled with tubercles of different ages. Especially in lungs, many of the tubercles were caseous with hard periphery. Pericardium filled with serous exudate.

6. Nineteen days after infection, a discharging nodule was found at point of inoculation. This failed to heal and later glands in both axillae and in one groin were found enlarged. Death on the eighty-seventh day. Caseous lymph glands in axillae and in groin. Spleen and liver large, hard, and full of tubercles and necrotic areas. Lungs filled with miliary tubercles. Pleural cavities filled with serous exudate.

7. Nodule found at site of inoculation on nineteenth day. Did not discharge and heal. Death 68 days after inoculation. Enlarged and caseous gland in axilla. Lungs, liver, and spleen filled with miliary tubercles. Very marked generalized tuberculosis.

8. Nineteen days after inoculation, a discharging nodule was found at site of inoculation. This later nearly healed, but several deeper glands in axillae enlarged

and became caseous. Death about four months after infection. Lungs, liver, and spleen full of tubercles.

The pills used in the above series contained from 2 to 3 mg. of selenium blue and a single pill constituted a daily dose. The next series was fed with pills containing each from 1.5 to 2 mg. of tellurium blue.

1. No local tubercle developed, but the pig became emaciated and died on the thirty-seventh day after infection. No enlarged glands in axilla, but lungs were filled with miliary tubercles; spleen and liver exhibited a few tubercles and a few small necrotic areas.

2. Eighteen days after infection, a discharging nodule was found at point of inoculation. This healed and deeper nodules were found 32 days after inoculation. Death on sixty-seventh day. Glands in both axillae slightly enlarged, with beginning caseation. Liver pale, lobulated, and showing several large areas of necrosis. Spleen large and necrotic with numerous tubercles of different sizes. Lungs filled with large, hard tubercles. Pericardium filled with cloudy, yellowish fluid.

3. Eighteen days after infection, a large hard nodule was found at site of injection. No healing. Other deeper nodules developed. Death 91 days after infection. Large ulcer in right axilla and under it the tissue was dense and granular. Spleen and liver large and filled with tubercles. Lungs packed with large opaque caseous tubercles. Pericardium filled with cloudy fluid.

4. Discharging nodule found in axilla on eighteenth day. This partly healed. Death about four months after inoculation. Spleen, liver, and lungs filled with tubercles.

5. Discharging nodule at site of injection found on eighteenth day. No healing. Death 94 days after inoculation. Raw ulcer on right side of chest wall. Enlarged and caseous glands in both axillae and in both groins. Lungs filled with miliary tubercles. Liver enlarged containing many yellowish-green patches of necrosis; spleen somewhat enlarged and necrotic along lower border.

6. Large, tense nodule found at point of inoculation on eighteenth day; this soon ruptured and partially healed. Deep nodules developed late. Death 90 days after infection. Ulcer and several caseous nodules in axilla. Spleen and liver enlarged and filled with tubercles and necrotic areas. Lungs filled with tubercles, many being caseous and others clear and hard.

7. A discharging nodule was found at site of injection on eighteenth day. This healed and deeper nodules developed. Death about four months after inoculation. Local glands enlarged and caseous. Liver, spleen, and lungs filled with tubercles.

8. Large tense nodule found at site of injection on eighteenth day. This discharged and healed. Death about four months after infection. Liver, spleen, and lungs filled with tubercles.

A comparison of the duration of life after infection of the treated as compared with the untreated animals and of the methylene blue as compared with the new dyes is summarized in Table 4.

All of the animals represented in this table received the same amount of the same culture of human tubercle bacilli and all the treated animals were fed with cake pills of the different dyes as before described.

TABLE 4.

Controls Days	Methylene Blue Days	Selenium Blue Days	Tellurium Blue Days
75	74	68	37
69	80	94	68
80	101	81	91
160	80	95	120
194	99	96	94
.....	113	87	90
.....	68	120
.....	120	120
Average 117.4	91½	88½	92½

CONCLUSIONS.

1. Methylene blue will penetrate the tubercle, stain the living tubercle bacillus, and in some cases kill the bacillus *in vitro* and in others lessen its virulence. When added to the culture media, a relatively small percentage of methylene blue will inhibit the growth of the human tubercle bacillus.

2. Methylene blue iodid is no less irritant than the chlorid and has less bactericidal power and no greater therapeutic value.

3. The new methylene blues are various modifications of the methylene blue molecule and have in the main no advantage over methylene blue. New methylene blue GG, however, showed some effect in the one case in which it was used therapeutically and it, with other oxygen derivatives of methylene blue, will be given further tests.

4. Selenium blue and tellurium blue are new blue dyes made in this laboratory, in which the sulfur of the methylene blue molecule is replaced by selenium and by tellurium. They are weaker and less stable dyes than methylene blue and more toxic and less bactericidal than that dye. They penetrate the tubercle and are reduced in it and can be reoxidized; they stain the living tubercle bacillus, but more faintly than does methylene blue. In fact, they behave in all respects as weaker editions of methylene blue and have no advantage over it.

5. Neither methylene blue nor any of the allied dyes tested by me may be said to have much therapeutic influence over experimental tuberculosis of the guinea-pig. While methylene blue seems for many reasons a favorable starting-point for tuberculosis chemotherapy, other modifications of it and probably many others must be tried before we can claim to have found a specific for this disease.

In closing, it gives me great pleasure to express my gratitude to Dr. Fraenkel for his intelligent and skilful co-operation with me in making the new dyes used in this work, and to Miss Sherman for her tireless assistance in all the experimental routine work.

THE EFFECT OF MEAT AND OF MEAT EXTRACT MEDIA UPON THE FERMENTATIVE ACTIVITY OF STREPTOCOCCI.*

JEAN BROADHURST.

(From the Veterinary College, Cornell University, Ithaca, New York.)

During the past few years several papers have appeared dealing with the fermentative reactions of streptococci in special media. The results obtained by different workers have proven discouragingly difficult to align on any common basis, such as origin. My own work now includes about 700 strains of streptococci from various sources, chiefly milk, blood, and the mucous membranes and the feces of animals, man included. Concerned, therefore, about this lack of correspondence, I sent a few throat strains to Professor C. N. Hilliard of Purdue University in order to check my own procedure. The strains were selected from throat strains on hand, and with reference to their raffinose-fermenting power, as our previous work showed a decided difference in the percentage of raffinose fermenters.

Duplicate subcultures of the strains chosen were incubated for 24 hours. One of each pair was sent to Professor Hilliard. Three days later we transferred our cultures to fresh agar slants (made with meat, not meat extract). Twenty-four hours later these fresh agar cultures were used to inoculate the Gordon media (sugar-free broth containing 1 per cent saccharose, lactose, salicin, raffinose, or mannite). The results (Table 1) proved very puzzling until Professor Hilliard¹ wrote that his Gordon media had been made from meat *extract*. For my Gordon media *meat* was used.

It will be noticed that all the substances fermented in the Gordon meat media show a decided falling-off in the Gordon meat extract

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¹ Professor Hilliard's records for meat extract are the averages of two duplicate Gordon tubes. My meat records are of single Gordon tubes, except 98 and 106, each of which gives the averages for two strains done in duplicate at the time of isolation and continued for six weeks as separate cultures; their titration records were practically alike. All cultures were incubated 3 days at 37°, and titrated in the cold, against n/20 phenolphthalein. The initial acidity has been subtracted from all results given in the tables.

media, as in Strain 81 where the raffinose titration record falls from 5.2 to 1.7, or in Strain 99 where the saccharose record of 2.8 falls to 0.7. Substances not at all or barely fermented in the meat media (-0.1 to 0.5) are not noticeably affected by the meat extract, though raffinose and inulin sometimes show a slight increase. (A slight increase is also seen in mannite for 106; earlier [January] records for 106 in mannite were 0.0.)

TABLE 1.
TITRATION RECORDS OF SEVEN THROAT STRAINS OF STREPTOCOCCI, FEBRUARY, 1913.

Strain	Source	Date Isolated	Medium	Sac- charose	Lac- tose	Sal- icin	Raf- finose	Man- nite
20.....	Throat, man; sus- pected diphtheria	Oct., 1912	Meat	3.5	3.8	4.0	2.5	2.7
			Meat extract	1.0	1.3	1.3	0.9	1.2
67.....	Palate, Cat 4	Jan., 1913	Meat	4.0	4.2	4.1	0.6	3.6
			Meat extract	1.3	1.8	1.3	0.0	1.6
81.....	Tonsils, Cat 4	" "	Meat	5.0	5.2	4.2	5.2	0.1
			Meat extract	2.3	2.3	1.8	1.7	0.2
98.....	Palate, Cat 6	" "	Meat	3.3	2.5	2.1	2.4	2.1
			Meat extract	0.8	0.8	0.7	0.7	0.8
99.....	Palate, Cat 6	" "	Meat	2.8	2.5	1.9	2.4	2.1
			Meat extract	0.7	0.9	0.7	0.7	0.8
100.....	Throat, Cat 1	" "	Meat	4.1	4.3	4.4	0.2	3.5
			Meat extract	1.0	1.8	1.9	0.0	1.5
106.....	Tonsils, Cat 6	" "	Meat	4.5	3.4	4.3	3.9	0.8
			Meat extract	1.6	1.8	1.4	2.3	1.4

For these different results the only explanation seemed to be the respective uses of meat and of meat extract. The initial acidity varied about 0.2, an insignificant matter if the fermentative activity can increase it to 5.2 as in 81, lactose (Table 1); Professor Hilliard's media had the *lower* and presumably more favorable initial acidity (0.3-0.5).

Later, to try out more fully these differences apparently caused by meat and by meat extract media, I selected from my stock cultures a series in which the strains differed in their original fermentative results, ranging from a non-fermenter (Strain 98, Table 2) to one that fermented six of the Gordon test media (Strain 71, Table 2). They varied also in the time under cultivation and in origin as described in the table. To subject the strains for a slightly longer period, contemporary subcultures were first made in plain broth, one lot from meat and the other from meat extract.

After 24 hours, corresponding meat and meat extract Gordon media were inoculated from the two sets of broth tubes. The conditions¹ were the same in every other respect. The titration results (Table 2) at the end of the usual time (3 days) showed practically the same differences as in the earlier series.

TABLE 2.
TITRATION RECORDS OF STREPTOCOCCI FROM VARIOUS SOURCES, SEPTEMBER, 1913.

Strain	Source	Date Isolated	Medium	Sac- charose	Lac- tose	Sali- cin	Raf- finose	Man- nite	Inulin
11.....	Milk	Oct., 1912	Meat	0.0	0.4	3.4	0.1	0.1	0.1
			Meat extract	0.1	0.5	1.7	0.3	0.2	0.2
.....	Intestine, dog	March, 1913	Meat	-0.1	3.6	3.0	-0.1	0.0	0.1
			Meat extract	0.2	1.6	1.8	0.3	0.1	0.2
9.....	Throat, man	" "	Meat	4.4	0.2	3.0	0.1	0.0	0.0
			Meat extract	1.8	0.1	1.6	1.4	0.0	0.0
42.....	Mastoid abscess, man	March, 1911	Meat	2.8	2.8	-0.1	2.5	0.1	0.3
			Meat extract	1.3	1.1	0.0	1.0	0.1	0.2
61.....	Milk	May, 1913	Meat	-0.1	3.3	0.0	0.2	0.3	0.0
			Meat extract	-0.1	1.2	0.1	0.1	0.2	0.2
71.....	Feces, equine	" "	Meat	3.3	3.2	3.8	2.5	2.9	2.3
			Meat extract	1.4	1.5	1.5	1.1	1.1	0.9
88.....	Feces, human	" "	Meat	0.0	3.1	3.8	0.1	2.9	0.0
			Meat extract	1.0	1.7	2.2	0.8	1.2	1.2
98.....	Throat, dog	" "	Meat	-0.1	0.0	0.0	0.0	0.0	0.0
			Meat extract	0.0	0.0	-0.1	-0.1	-0.1	0.0
103.....	Milk	June, 1913	Meat	3.4	3.3	2.4	0.0*	0.0	0.0
			Meat extract	1.4	1.4	1.0	1.0	0.2	0.5
111.....	Anthrax blood	" "	Meat	0.6*	2.7	3.1	0.2	2.3	0.1
			Meat extract	0.8	0.9	1.3	0.8	0.8	0.8
233.....	Duodenum, dog	August, 1913	Meat	2.9	2.8	2.9	0.1	0.1	-0.1
			Meat extract	1.2	1.0	1.0	0.6	0.3	0.6

* Previously fermented; titration record of 103 raffinose=3.4; of 111 saccharose=1.8.

Smears were made of all my Gordon media before titration. In this preliminary report it is sufficient to state that later examination testified to the purity² of the cultures, and indicated that physiological differences might be expected. Slight but not always parallel differences in the gross appearances of both the plain broth and the Gordon media were noted. These, too, do not materially affect the significance of the titration results.

¹ The same stock of substances (peptone, inulin, etc.) was used throughout. The only difference, as before stated, was that meat (15 pounds to 13 liters) was used in one case, and meat extract (3 gm. to 1 liter) in the second case. The sugar-free media (*B. coli*, 7 days at 37°) were filtered through a Berkefeld filter. All the media used were sterilized in the Arnold, not in the autoclav, and for like periods of time.

² Before inoculation, to insure sterility, all of the Gordon tubes were incubated at 37° for one day; they had been sterilized but 30 minutes on two consecutive days.

The depression of the fermentative activities is evidently not permanent. A third short series (not given here), in which the organisms were kept for six days (two transfers) in meat and in meat extract broth and then incubated in Gordon media made of meat, showed no final titration differences.

Publication of data based on so few strains is justified, I feel, in this case by two facts: First, the differences are not only most marked (often reaching 200 to 300 per cent), but they are remarkably uniform in amount and direction. Second, other and longer series to test constancy and the influence of age, temperature, and the kind of medium show no such marked or uniform effect.

In the last few years a number of bacteriologists have been interested in the fermentative powers of the streptococci; several papers on this subject have appeared, and other papers, read during the last year in New York and Washington, have not yet found their way into print. The difficulty one finds in lining up his work with that of the other workers is due, I suspect, to the fact that some have used meat, others meat extract, in making up their media. Litmus and phenolphthalein, too, further complicate the end results. With meat media it would make less difference which indicator was used. But in meat extract media, where the final acidity record often drops down to 0.7-1.0, litmus with its higher neutral point gives no indication of the possible powers of the strain under investigation.

CONCLUSIONS.

The fermentative activities of streptococci vary greatly with the use of meat or of meat extract in making the special media. All workers should, therefore, state definitely whether meat or meat extract media were used throughout such investigations.

Qualitative results, estimated with litmus as an indicator, are not comparable with quantitative ones, with phenolphthalein as an indicator, if meat extract is used in making the special media.

THE INFLUENCE OF STRYCHNIN, CAFFEIN, CHLORAL, ANTIPYRIN, CHOLESTEROL, AND LACTIC ACID ON PHAGOCYTOSIS.*

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There are three essential factors concerned in bacterial phagocytosis on which chemical substances may act so as to increase or depress phagocytic activity, namely: (1) the leukocytes, (2) the opsonins, and (3) the bacteria.

The effects of a variety of substances on phagocytosis have been studied and a brief statement of the results may be of interest.

THE ACTION OF SALTS ON PHAGOCYTOSIS.

That a large number of salts inhibits phagocytosis was demonstrated by Hektoen and Ruediger, who found that CaCl_2 , BaCl_2 , SrCl_2 , MgCl_2 , K_2SO_4 , NaHCO_3 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $\text{Na}_2\text{C}_2\text{O}_4$, $\text{K}_4\text{Fe}(\text{CN})_6$ all neutralize or bind the opsonins so that these cannot act on the bacteria. Eggers studied the effects of salts normally present in the serum and found that BaCl_2 and NaF have a marked toxic action on the leukocytes. MgCl_2 has a stimulating action on the leukocytes.

Calcium has a distinct stimulating action on phagocytosis of carbon particles as shown by Hamburger. A 0.005 per cent solution causes a rise of phagocytosis of 22 per cent (calcium chlorid). Furthermore, calcium has a marked positive chemotactic action, increasing the movements of the phagocytes not only *in vitro* but also in the living body. The action appears to be a specific property of this element and is not dependent on its electrical charge.

THE EFFECT OF REACTION.

One would expect opsonins to exert their maximum activity in normal serum, i.e., in a slightly alkaline medium. Hamburger and Hekma found that a very slight acid reaction is sufficient to inhibit phagocytosis to a marked degree. When the reduction of the OH ions reached 5 per cent or when they were increased 15 per cent there was a marked effect. Noguchi, however, claims that normal opsonins act best in neutral reaction and that no opsonization occurs in a serum containing more alkali than 1.6 c.c. of N/20, or more acid than 0.5 c.c. of N/20 per 1 c.c. of serum. Eggers has found that the maximum action occurs at normal (alkaline) reaction of the serum, agreeing with Hamburger and Hekma.

THE EFFECT OF ANTISEPTICS.

The action of some surgical antiseptics on phagocytosis was studied by Manwaring and Ruh. Carbolic acid causes from the first a decrease of phagocytosis, with cessation at 0.5 per cent. Mercuric chlorid in weak solution (less than 1/120 per cent)

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causes transient stimulation followed by depression. Neuber found that mercuric chlorid injected into animals has the same effect. Hektoen and Ruediger showed that formalin causes marked inhibition of phagocytosis in solution of 1 to 5,000.

I have found that sodium iodoxybenzoate has a marked stimulating action on the phagocytosis of streptococcus and staphylococcus by human leukocytes in the presence of human serum. This substance has a distinct germicidal action which is dependent on the oxygen attached to iodine in the molecule. Likewise, the stimulating effect on phagocytosis is due to the oxygen, since sodium iodbenzoate, which contains no oxygen, has no effect. The iodoxybenzoate probably acts on the opsonin to produce a more active combination. That the oxygen is the important factor is also indicated by the fact that potassium cyanide, which depresses oxidation, has a marked inhibitory influence on phagocytosis *in vitro*. These experiments suggest a relation-ship between oxidation and the process of phagocytosis.

THE ACTION OF RADIUM ON PHAGOCYTOSIS.

Chambers and Russ found that the alpha and beta rays from comparatively small quantities of radium have a direct bactericidal action. The polymorphonuclear leukocytes of human blood suffer a reduction in their phagocytic power and are eventually destroyed when exposed to the alpha rays. The opsonin in normal serum is destroyed by these rays. Reiter found that the emanations *in vitro* stimulate the phagocytic activity of the blood cells in some experiments to 30 per cent. Under certain circumstances the emanations may so act on the bacteria as to protect them from phagocytosis.

Bouchard, P. Curie, and Balthazard found that a leukopenia occurs in animals poisoned with radium emanations. Klecki recently found that radium emanations have a different effect on the phagocytosis of different bacteria. Radium increases the phagocytosis of the colon bacillus and the staphylococcus, and reduces the phagocytosis of the tubercle bacillus. The increase of phagocytosis is chiefly through the action on the phagocytes, and to a lesser degree on the bacteria.

ACTION OF COLLOIDAL METALS.

Bossan and Marcelet found that colloidal suspensions of gold, copper, silver, and platinum have a marked stimulating action on the production of opsonins, as well as on phagocytosis *in vitro*. They assert that there is also a certain specificity of action. Silver and gold are very active toward paratyphoid, copper toward the cholera vibrio, and platinum toward the streptococcus. This specificity is very interesting in view of the results which I have obtained on the germicidal action of oxidizing substances. I found that sodium iodoxybenzoate has different bactericidal powers against different organisms, being most active toward those organisms containing the least catalase. The catalase value seems to explain the specificity in the organisms which I studied. This work will soon be published. Since the colloidal metals are active oxidizing agents their effect on phagocytosis may be due to this property, just as the effect of sodium iodoxybenzoate is due to the oxygen in the molecule.

Collargol causes a leukocytosis with increase in the polymorphonuclears (Dunger). This leukocytosis is not accompanied by any increase in phagocytosis (Hoffman). According to Hamburger and Hekma colloidal silver (Crede) has no effect on phagocytosis *in vitro*. The colloidal solutions of various metals, as manganese, iron, copper, platinum, silver, gold, etc., exhibit properties closely resembling those of the catalases.

A. and L. Lumière and Chevrotier have confirmed the work of Trillat on the power of colloidal solutions of manganese to effect oxidations. According to these authors colloidal solutions of any metal capable of existing in two or more states of oxidation should exhibit properties similar to those of oxidases, the state of division of the colloidal substance determining the oxidizing power. Emulsions of iron or of manganese in albumen, gelatin, or gum have the power to blue guaiac, oxidize hydroquinon, pyrogallol, and paraphenyldiamin. They also found that the artificial oxidases protected animals against tetanus toxin. The work of Klebs, Linossier, Meyer, Bertrand, etc., makes it evident that certain metals, Fe, Mn, and Cu, enter into the composition of the oxygen-carriers of the living organism as essential ingredients. Robin and Bordet have arrived at the conclusion that the colloidal metals, especially manganese, promote oxidation in the tissues and intensify metabolism.

The bactericidal action of the colloidal metals is quite uncertain, as various investigators have obtained different results. M. Ascoli and G. Izar have given us an insight into the action of the metal hydrosols. Small amounts of the silver hydrosol activate the diastatic ferment of the liver and blood serum. Ascoli has further found that the metal hydrosols increase autolysis to varying degrees. This action can be inhibited by traces of HCN, HgCl_2 , and CO, just as is their property to split H_2O_2 . The colloidal metals in stable condition increase the nitrogen exchange, especially the nuclein metabolism (M. Ascoli and G. Izar). On intravenous injection silver hydrosol is found chiefly in the liver, bone marrow, and spleen. It is taken up by the Kupffer cells in the greatest amount.

ARSENICAL COMPOUNDS.

The investigations of Friedberger and Masuda indicate that the agglutinins are increased by salvarsan. The normal hemolysin is also increased. It is asserted that salvarsan noticeably increases the opsonin also two to eight hours after intravenous injection (Strubell). Boehncke found that salvarsan stimulates the production of antibodies. These results agree with those of Agazzi that various arsenic compounds stimulate antibody formation, and confirm the prediction of Ehrlich that arsenobenzol would stimulate the antibody-producing organs.

ALKALOIDS.

Only a few alkaloids have been studied for their effect on immune reactions. These are quinin, morphin, curare, and atropin. Manwaring and Ruh and Thomas Wilson found that quinin causes stimulation of phagocytosis in weak solution. According to Grünspan it has a slight stimulating effect in dilution of 0.002 per cent. Morphin has a depressing action (Reynolds). Cantacuzene demonstrated a detrimental action of opium on leukocytes and found that animals were rendered more susceptible to typhoid when given opium. Platania in 1889 found that frogs injected with curare were unable to overcome the subcutaneous injection of anthrax to which they are normally resistant.

ANESTHETICS AND Soporifics.

Alcohol, chloroform, and ether have a detrimental action toward the natural defenses against infection (Rubin). They suspend phagocytosis *in vitro* (Snel). Ether reduces the property of phagocytosis in human blood (Graham). Laitinen found that alcohol hastens the death of experimentally infected animals. Similar results were obtained by Zimin, Abbot, and Krutschilin, for ether, chloroform, and

alcohol. Snel found that chloral renders animals more susceptible to infection, but he did not study its effect *in vitro*.

The importance of the fats and lipoids in immune processes has been clearly demonstrated by Kyes, Neuberg, Morgenroth and Carpi, Woelfel, Vay, Noguchi, and others. Noguchi has even suggested that some of the complements of the serum may be salts of the higher fatty acids with weak organic acids. Hence substances which are solvents of those lipoids may be expected to have a marked effect on the immune reactions, as is the case with alcohol, ether, chloroform, etc. Graham found that ether given for anesthetic purposes reduces the property of phagocytosis, because of a direct action of the ether on the serum and the leukocytes. This action of the ether is probably due to its fat-solvent power. Lecithin counteracts this inhibitory effect of ether both *in vitro* and when given subcutaneously.

Likewise, as pointed out by Wells, the substances which precipitate fatty acids, such as calcium, barium, and strontium ions, inactivate the complement.

THE ACTION OF ANTIPYRETICS.

The action of antipyretics on phagocytosis has been investigated by Kentzler and V. Benczur. Quinin, antipyrin, phenacetin, pyramidon, and sodium salicylate have practically no effect. Quinin in typhoid, antipyrin in influenza, phenacetin in parametritis, pyramidon in pneumonia, and sodium salicylate in tonsillitis do not affect the phagocytic index.

LECITHIN AND CHOLESTEROL.

Kyes demonstrated that the hemolytic constituent of cobra venom could be complemented by lecithin. Morgenroth and Carpi found the same to be true for bee poison. Graham found that lecithin counteracts the inhibitory effect of ether on phagocytosis; it cannot act as opsonin in salt solution nor is it able to reactivate serum whose complement has been destroyed by heat. Walbum, who used a colloidal suspension, found cholesterol to have a marked stimulating effect on phagocytosis. I have repeated the experiments *in vitro* but with opposite results, as given below.

Finally Bechhold has studied the effect of some organic colloids on the serum (gelatin, peptone, dextrin, hemoglobin, egg albumin, pepsin, and pancreatin). Peptone is a very active stimulant of the leukocytes in a dilution of 1/160,000. Lactic acid in relatively large amount (n/40) causes destruction of the leukocytes. In dilutions above n/200 it seems to have no effect on phagocytosis in salt solution, serum, or whole blood. Bechhold found that lactic acid stimulated the phagocytosis of staphylococcus, and that both pepsin and pancreatin have a stimulating action in the presence of lactic acid. These results are not in accord with our observations on lactic acid, given below.

Neisser and Guerrini observed that a series of substances, toxic in large amount, are in small amount stimulants of the leukocytes. These so-called "leuko-stimulantien" are peptone (1/160,000), nucleic acid (1/100), quinin (1/12,800), potassium iodid (1/1,000), staphylolysin (1/500), alcohol (1/512). The leuko-stimulation occurs not only in immune serum but also in normal serum and even in the absence of opsonins. Peptone is the most active of these stimulants. Staphylolysin, a leukocyte poison, is in small amounts a stimulant of phagocytosis. Perhaps the bacteria, by the production of such substances, contribute to their own destruction. Furthermore, these results suggest that the stimulating action of some immune sera may be due to the presence not only of opsonins but also of leuko-stimulants, such as nucleic acid, peptone, etc.

TECHNIC OF STUDYING PHAGOCYTOSIS IN THE EXPERIMENTS NOW RECORDED.

The method employed in this work is essentially that of Wright and Douglas, with changes necessitated by the study of the action of the chemical substances on the process. Equal parts of human serum and the substance studied, and washed human leukocytes and the suspension of the bacterium studied are mixed and incubated at 37° C. for 15 minutes. Smears are then made in the same order as the mixtures in the pipettes were prepared, thus equalizing the time of action of the mixtures as much as possible. The number in any series is usually below eight or ten, thus making the time necessary for the preparation of the smears as short as possible. Controls, consisting of equal parts of human serum, and physiological salt solution in place of the drug studied, and washed human leukocytes, and the same suspension of the bacterium studied, are made at the beginning and at the end of each series. The average of these two counts is taken as the control count. The smears are made and stained with methyl-thionin. An average of 100 leukocytes is counted from each smear.

THE ACTION OF STRYCHNIN.

The fact that strychnin has a marked stimulating action on most animal organisms, and is widely used in various infectious diseases, led to the study of its action on phagocytosis. Strychnin has been shown to be less powerful in its action on leukocytes than any of the cinchona alkaloids. Strychnin has only one-fourth of the action of quinin toward infusoria. It accelerates the movements of medusae, and stimulates somewhat the movements of mollusca, but never causes any general tetanus in this animal. Its action in the vertebrates is well known.

Strychnin greatly increases the consumption of oxygen and the output of carbon dioxid, even when the muscular contraction has been previously eliminated by curare. The augmentation of oxidation in the tissues is accompanied by an increased formation of heat. Whether its stimulating action on phagocytosis, as shown by the following experiments, is due to its influence on oxidation, is difficult to determine, but the fact that alkaloids which depress oxidation also depress phagocytosis makes this a probable explanation.

The action of strychnin sulfate and nitrate on phagocytosis of streptococcus by human leukocytes was studied *in vitro*. The various dilutions of the salt were made in physiological salt solution. Further, leukocytes were treated with strychnin solution, washed free from the strychnin, and compared with untreated leukocytes. Finally, dilution experiments were carried out, using strychnin to

dilute the serum in one series and salt solution in the other, and the dilution at which phagocytosis equals the spontaneous phagocytosis determined.

Serum + salt solution				+ leukocytes	+ streptococcus	4.5
" + 1/10 per cent strychnin sulfate	+	"	+	"	+	"1.5
" + 1/25 " " " "	+	"	+	"	+	"4.35
" + 1/75 " " " "	+	"	+	"	+	"6.24
" + 1/100 " " " "	+	"	+	"	+	"7.97
Serum + salt solution				+ leukocytes	+ streptococcus	3.0
" + 1/250 per cent strychnin sulfate	+	"	+	"	+	"4.5
" + 1/500 " " " "	+	"	+	"	+	"5.7
" + 1/1000 " " " "	+	"	+	"	+	"5.2
" + 1/2000 " " " "	+	"	+	"	+	"5.2

These results indicate that strychnin sulfate has a stimulating action on phagocytosis of streptococcus by human leukocytes in the presence of human serum. The action is probably on the opsonin of the serum, possibly by the formation of a more active opsonin-strychnin combination. That the action is not on the leukocytes is indicated by the following experiment:

Serum + leukocytic suspension treated with 1/500 per cent strychnin sulfate in salt solution for 20 minutes at 35° C., centrifuged, washed with salt solution + streptococcus:							
Phagocytic index	I.5					
Salt solution + leukocytic suspension (as above):							
Phagocytic index	0.36					
Serum + leukocytic suspension treated with salt solution only (as above):							
Phagocytic index	I.4					
Salt solution + leukocytic suspension treated with salt solution only (as above):							
Phagocytic index	0.34					

Here we see that the phagocytic index with leukocytes treated with strychnin sulfate was the same as that with leukocytes not treated with strychnin solution, indicating that there is no action on the leukocytes in the solution used. That the action is on the serum is indicated by the results of dilution experiments:

Salt Solution Dilutions.		Strychnin Sulfate Dilutions (1/500 per cent).	
SerumI.0	SerumI.0
Serum /40.52	Serum /40.82
Serum /80.32	Serum /80.56
Serum /320.28	Serum /320.36
Serum /640.0	Serum /1280.18
Salt solution0.26	Salt solution0.20

Hence with strychnin sulfate the dilution required to stop phagocytosis is greater than with physiological salt solution, indicating that the action is on the opsonin of the serum.

A series of experiments with strychnin nitrate shows a similar stimulating action on phagocytosis. Here also the action is on the serum and not on the leukocytes. In the absence of serum the strychnin has practically no action. Since the mixtures in these experiments consist of four equal parts, the dilution of the strychnin solution is four times that of the solution used. Hence the strychnin sulfate has its most marked stimulating effect at 1/2000 per cent and the action of 1/8000 per cent is almost as great. This action of strychnin offers a possible explanation of its value in various infectious diseases and suggests a more extended use of its preparations in such diseases.

THE ACTION OF MORPHIN.

The following experiments were made with morphin to test its effect on phagocytosis:

Serum + salt solution	+	leukocytes	+	streptococci	4.70
Salt solution + salt solution	+	"	+	"	0.06
Serum + 1 per cent morphin sulfate	+	"	+	"	2.02
" + 1/10 " " "	+	"	+	"	2.36
" + 1/50 " " "	+	"	+	"	3.98
" + 1/100 " " "	+	"	+	"	4.02
" + 1/1000 " " "	+	"	+	"	4.40
Serum + leukocytic suspension treated with 1/10 per cent morphin sulfate in salt solution for 30 minutes at 35° C., centrifuged, washed with salt solution + streptococci:						
Phagocytic index						8.1
Serum + leukocytic suspension treated with salt solution only as above + streptococci:						
Phagocytic index						12.7

These results indicate that morphin sulfate has a depressing action on phagocytosis of streptococci by human leukocytes up to 1/100 per cent and to a slight degree up to 1/1000 per cent. This action is largely on the leukocytes, for leukocytes treated with 1/10 per cent morphin sulfate gave an index of 0.63 as compared with 1.0 for leukocytes treated only with salt solution. That this depressing action is of considerable importance in infection has

already been demonstrated by animal experimentation. Animals given opium in various forms, as well as morphin itself, succumb more readily to anthrax and other infectious diseases than normal animals (Cantacuzene, Oppel, and Gheorghiewski).

THE ACTION OF CAFFEIN.

In the study of the action of caffein the pure alkaloid was used, as it, unlike most of the alkaloids, is soluble in water. All of the dilutions were made in normal salt solution:

Serum + salt solution				+ leukocytes + streptococci	2.8
Salt solution + salt solution	+	"	+	"	0.06
Serum + caffein 1 per cent	+	"	+	"	0.36
" + " 1/10 "	+	"	+	"	2.46
" + " 1/100 "	+	"	+	"	2.74
" + " 1/500 "	+	"	+	"	2.94
" + " 1/1000 "	+	"	+	"	2.70
" + " 1/2000 "	+	"	+	"	2.70
Serum + salt solution				+ leukocytes + streptococci	3.64
Salt solution + salt solution	+	"	+	"	0.2
" " + caffein 1/100 per cent	+	"	+	+ leukocytes + streptococci	0.2
" " + " 1/500 "	+	"	+	"	0.26
" " + " 1/1000 "	+	"	+	"	0.1

The foregoing results indicate that caffein has a depressant effect on phagocytosis up to 1/10 per cent. In dilutions greater than this it has practically no action, there being no effect on the leukocytes or on the opsonin of the serum. The widespread use of preparations of caffein as beverages makes it a substance of considerable interest. Caffein, unlike alcohol, ether, opium, strychnin, etc., induces no marked symptoms in moderate quantities, and unlike these it has no marked effect on phagocytosis.

THE ACTION OF CHLORAL.

Chloral has the same general action as the anesthetics, hence it is interesting to learn its effect on phagocytosis and compare it with the action of the anesthetics. The effects of chloral on the tissues have been found to correspond closely to those of chloroform. Imperfect oxidation is produced, accompanied by an increase in unoxidized sulfur in the urine; less oxygen is absorbed and less carbon dioxid excreted (Cushny).

Like chloroform, chloral has antiseptic properties, produces fatty changes, and causes depression; unlike chloroform, it has a local irritant action, and causes no stage of stimulation.

The results with chloral follow:

Serum + salt solution				+ leukocytes + streptococci	1.7
Salt solution + salt solution				+ " + "	0.2
Serum + chloral 1/50 per cent				+ " + "	1.0
" + " 1/100 "				+ " + "	1.3
" + " 1/500 "				+ " + "	1.4

Hence chloral hydrate in a dilution of 1 to 5,000 has a distinct inhibitory effect on phagocytosis and this is present even in 1 to 50,000. It may, therefore, be of importance in infections not to prescribe large amounts of chloral, for by so doing the phagocytic power may be reduced, as has already been demonstrated by Snel for infected animals.

THE ACTION OF CHOLESTEROL.

I have repeated the experiments of Walbum, using the colloidal suspension of cholesterol made according to Porges and Neubauer, i.e., first dissolving the cholesterol in acetone and gradually adding the acetone solution, with constant stirring, to the required amount of distilled water. The acetone is removed by evaporation at a low temperature, and the suspension then filtered. The suspension used was N/2000, as employed by Walbum (0.193 gm. per liter).

Serum + leukocytes + streptococci	2.74
Cholesterol + leukocytes + streptococci	1.54
Salt solution + leukocytes + streptococci	0.52
Serum + cholesterol + leukocytes + streptococci	1.18
Salt solution + cholesterol + leukocytes + streptococci	1.94

These results indicate that cholesterol has an inhibitory action on phagocytosis in the presence of serum *in vitro*. This can be explained by action on the serum, the cholesterol probably combining with the opsonin as it is known to combine with various lysins. This combination is less active than the cholesterol itself. For we see in the above experiments that cholesterol is capable of replacing the opsonin of the serum to a certain extent, cholesterol giving a higher phagocytic index than salt solution. It does not

completely replace the opsonin in the dilutions used in these experiments; the index of the serum is higher than with the colloidal cholesterol alone.

That this action of cholesterol may be due to its colloidal condition cannot be denied, altho, according to Walbum, colloidal suspensions of the aliphatic alcohols, cetyl, and myricyl alcohol are inactive. Walbum found that cholesterol gave a higher phagocytic index with the colon bacillus than normal human serum. This is not in accord with our results with the streptococcus.

THE ACTION OF ANTIPYRIN.

Serum + salt solution				+ leukocytes + streptococci	3.1
Salt solution + salt solution				+ " + "	0.4
Serum + antipyrin 1	per cent			+ " + "	2.6
" + "	1/10	"		+ " + "	3.4
" + "	1/50	"		+ " + "	3.7
" + "	1/100	"		+ " + "	3.2

These results indicate that antipyrin has a slight stimulating action on phagocytosis in dilutions of 1/10 to 1/50 per cent. Above this dilution it has no effect. It has been observed by Schultze and Beniasch that the use of the antipyretics does not retard the formation of the protective substances, for in infected animals treated with enormous quantities of antipyrin the serum displayed the same agglutinating properties as that of the control animals not subjected to the drug. In this connection may be mentioned the results of Kentzler and v. Benczur, who found that the antipyretics in solutions of 1/100, 1/500, 1/1,000 have practically no effect on the phagocytosis of staphylococci *in vitro*. In animals and in man they cause a slight and transient rise of phagocytosis or no effect at all.

THE ACTION OF LACTIC ACID.

In certain experiments, the records of which were placed at my disposal by Dr. Hektoen, lactic acid appears to suspend hemolysis and phagocytosis by acting on the complement and the opsonin. Thus 0.001 c.c. of a concentrated lactic acid (77.14 per cent $C_3H_6O_3$ by weight) prevents all hemolytic effect of 0.2 c.c. of dog serum upon guinea-pig corpuscles (total quantity in each tube being

2 c.c.); and 0.0006 of this acid neutralizes the double lytic dose of normal guinea-pig serum (complement) for 1 c.c. of a 5 per cent suspension of washed rabbit corpuscles sensitized in heated, immune, guinea-pig serum. The dilution of lactic acid in the above experiments is 1 in 2,596 and 1 in 4,320.

Lactic acid in doses of 0.003-0.0015 prevents phagocytosis of anthrax bacilli and staphylococci by the leukocytes in 0.5 c.c. of human blood. The result of the following experiments indicates that this action is due to neutralization or destruction of the opsonin. 0.2 c.c. of serum is mixed with 0.0015 c.c. of lactic acid in 0.5 c.c. of salt solution and placed at 36° C. for one hour; anthrax bacilli are then added and the tube returned to the incubator for 30 minutes; at this time the bacilli are washed twice and suspended in 0.5 c.c. of salt solution to which is added 0.5 c.c. of a suspension of homologous washed corpuscles. As control, mixtures prepared in the same way, except that the bacilli are treated with serum free from lactic acid, are used. Counting 50 leukocytes in each case with special reference to phagocytosis gives the following result:

	PHAGOCYTOSIS	
	+	o
Bacilli sensitized in dog serum + lactic acid.....	0	50
" " " " " + NaCl only.....	45	5
" " " human serum + lactic acid.....	0	50
" " " " " + NaCl only.....	38	12

Clearly the lactic acid in some way prevents the serum from making the bacilli susceptible to phagocytosis. In full accord with the anti-phagocytic effect of lactic acid is its neutralization of the anthracidal action of defibrinated dog blood as shown in the following table:

DEFIBRINATED DOG BLOOD 0.5 C.C. + LACTIC ACID + NaCl SOLUTION TO MAKE 1 C.C.	NO. OF BACILLI IN TOTAL QUANTITY		
	At Once	3 Hrs.	6 Hrs.
Lactic acid 0.003.....	5,880	0	0
" " 0.0015.....	"	800	4,270
" " 0.00075.....	"	6,370	3,080
" " 0.000375.....	"	600	88
NaCl only + blood.....	"	548	22

SUMMARY.

From the results of experiments recorded in the literature and the results I have obtained it appears that the effect of chemical substances on phagocytosis varies with their chemical constitution and their pharmacological action.

Alcohol, chloroform, ether, chloral, morphin, and potassium cyanid depress phagocytosis *in vitro*. The substances all have an inhibitory effect on oxidation, to which their action on phagocytosis is in all probability due.

That oxidative processes play a rôle in phagocytosis is definitely shown by the fact that sodium iodoxybenzoate, which is an organic peroxid and owes its pharmacological action as well as germicidal action to the presence of physiologically active oxygen attached to the iodine atom in its molecule, has a marked stimulating effect on the phagocytosis of streptococci and staphylococci *in vitro*, as I have previously demonstrated. The action appears to be on the opsonin of the serum, which is rendered more active. Likewise strychnin and certain colloidal metals which have a stimulating effect on oxidative processes stimulate phagocytosis to a marked degree, as shown in this report. The beneficial effects of improved oxygenation of the blood in infectious diseases may be due in part to the stimulating action on phagocytosis. Furthermore, substances which have a depressing effect on oxidations, as potassium cyanid, chloroform, ether, morphin, germicides, etc., have a distinct detrimental effect on phagocytosis as well as other immune reactions.

Caffeine and the antipyretics which are more inert drugs have little, if any, action on phagocytosis. They have no known effect on oxidations in the body.

The salts are mostly injurious to phagocytosis, to an extent depending on their toxicity, their ability to effect an exchange of ions with the salts in the cells, and their ability to combine with the opsonins.

Those substances which depress phagocytosis *in vitro* have been found, when studied *in vivo*, to have a detrimental effect on the immunity reactions, e.g., the anesthetics, chloral, morphin, some salts, and the antiseptics. The substances which have been found

to stimulate phagocytosis at all are calcium, magnesium, and mercuric chlorids; quinin; colloidal metals; peptone in dilute solution; nucleic acid; a few other leuko-stimulants such as staphylolysin in weak solution, potassium iodid, and perhaps alcohol; strychnin sulfate and nitrate; sodium iodoxybenzoate; salvarsan and other arsenic compounds; and possibly certain colloidal suspensions of cholesterol. It is interesting that all of these which have been studied *in vivo* have also had a favorable action, namely, salvarsan, calcium chlorid, quinin, colloidal metals (platinum, silver, gold, copper), mercuric chlorid in weak solutions, sodium iodoxybenzoate (results not yet published), and cholesterol in colloidal suspension (Walbum). Hence the action of a chemical substance on phagocytosis *in vitro* is a good index of the effect of that substance when used *in vivo*.

The mechanism of the action of chemical substances on opsonin has never been carefully worked out. It has been shown conclusively that the opsonic power of a serum is dependent upon a thermostable element, the activity of which is greatly increased by a coexistent thermolabile complement-like substance. This is true of normal as well as of immune serum; in the latter the thermostable element is increased. Whether the chemical substances act on the specific thermostable element which unites firmly with the object of its action, or on the thermolabile element, remains to be determined.

Strychnin has a marked stimulating action on the phagocytosis of streptococci by human leukocytes in the presence of human serum. The stimulating action is probably on the opsonin of the serum by the formation of a more active opsonin-strychnin combination. Whether the action is on both elements of the opsonin or on either one remains to be determined. The action of strychnin on phagocytosis offers an explanation of its value in infections, and suggests a more extended use of this drug. The interaction of the specific antibody and the chemical substance is in this case a beneficial one, and emphasizes the possibility of a combined therapy against the infectious diseases, the antibodies produced in the body being increased in amount by drugs introduced from without or their action on the invading microorganism accelerated.

Morphin has a depressing effect on the phagocytosis of streptococci by human leukocytes in the presence of human serum. The effect is largely on the leukocytes. As is generally assumed, large doses of morphin lower the resistance to infection, hence it should be used cautiously in bacterial diseases.

Caffein has practically no effect on the phagocytosis of streptococci by human leukocytes in the presence of human serum, in dilutions greater than 1/10 per cent. Stronger solutions have an inhibitory effect. It is interesting that this alkaloid, unlike morphin or strychnin, produces no marked symptoms when given internally in moderate quantities, nor has it any effect on oxidative processes.

Chloral has a distinct inhibitory effect on the phagocytosis of streptococci by human leukocytes in the presence of human serum. That this detrimental action is of importance in infection has already been demonstrated.

Cholesterol in colloidal suspension is capable of replacing the opsonin of the serum, but not completely, in the suspension used (n/2,000). It causes some phagocytosis of streptococci by human leukocytes in the absence of serum. The phagocytosis is not as great as that produced by the normal serum. Cholesterol reduces the opsonic power of normal serum, probably by combining with the opsonin. This combination of cholesterol with serum is less active than either the serum alone or the cholesterol alone.

Antipyrin has no effect on the phagocytosis of streptococci by human leukocytes in the presence of human serum. The antipyretics have been shown to have no effect on the formation of protective substances *in vivo*.

Lactic acid suspends hemolysis and phagocytosis by acting on the complement and the opsonin. This action occurs in dilutions of 1-2,000 to 1-4,000. The lactic acid prevents the serum from making the bacilli (anthrax) susceptible to phagocytosis. Lactic acid has not only an antiphagocytic effect but it also neutralizes the anthracidal action of defibrinated dog blood.

The effects of an agent on the processes of immunity should be considered carefully when the use of that agent seems indicated in infectious diseases. The possibility of a combined therapy, con-

sisting of the use of immune sera or vaccines together with certain drugs, suggests itself in the case of drugs which have themselves a stimulating effect on phagocytosis. The drugs may act as leukostimulants, or increase the production of antibodies, or combine with the antibodies to render them more active.

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OBSERVATIONS ON THE RELATIVE CONSTANCY OF AMMONIA PRODUCTION BY CERTAIN BACTERIA.*

STUDIES IN BACTERIAL METABOLISM. X.

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Experiments were reported in a series of previous communications¹ which were designed to measure quantitatively the nature and extent to which utilizable carbohydrate protects protein or protein derivatives from bacterial breakdown. The principle upon which this sparing action of carbohydrate for protein depends is of more than academic interest: it plays a prominent part in many practical as well as theoretical fields in which bacterial activity is involved. This principle, which is fundamentally concerned in the metabolism of utilizable carbohydrate and protein by bacteria, may be stated thus: "Fermentation takes precedence over putrefaction,"² that is to say, those bacteria which can utilize both carbohydrate and protein utilize the former in preference to the latter when both are simultaneously available. It should be remembered in this connection that some protein must be available, even when carbohydrate is being thus acted upon, since bacteria in common with all known cellular organisms contain nitrogen in their substance. Carbohydrate contains no nitrogen, consequently carbohydrate alone is obviously unable to supply the nitrogen. Bacteria cannot grow in pure carbohydrate solutions. This nitrogen requirement is minimal when bacteria are metabolizing carbohydrate. That it is indispensable, however, focuses attention sharply upon two entirely distinct activities which the bacteria, and indeed all living cells, exhibit, the structural (anabolic) and the vegetative (katabolic) functions. The structural function precedes the vegetative function chronologically, for the cell must be formed before it can carry on its appropriate functions. The

* Received for publication June 18, 1913.

¹ Kendall and Farmer, *Jour. Biol. Chem.*, 1912, 12, pp. 13, 215, 219, 465; *ibid.*, 13, p. 63.

² Kendall, *Jour. Med. Research*, 1911, 25, p. 117.

actual amount of material involved in the structural process is very little; it can be estimated with a fair degree of accuracy. The following simple calculation indicates the manner in which this estimation is reached. The colon bacillus is approximately one micron in diameter and two microns long. Its volume is, consequently, one micron \times one micron \times two microns. Assuming for simplicity of calculation that it is a rectangular prism instead of a cylinder, the volume of a colon bacillus is $0.001 \times 0.001 \times 0.002$ mm. The specific gravity of the colon bacillus has been estimated at 1.038, hence the weight of a single colon bacillus would be theoretically: $0.001 \times 0.001 \times 0.002 \times 0.038$, or 0.000,000,0021 mg. That is to say, two thousand million colon bacilli would weigh about one milligram. Eighty-five per cent of this weight is water. Hence the actual weight of organic material is inconceivably small. It should be remembered in this connection that the structural requirement, aside from losses incidental to enzyme formation, etc., which must be replaced, is ended when the colon bacillus is morphologically complete. On the contrary the requirements for vegetative activity, the "fuel" requirement in other words, is a continuous one which ceases only when the organism dies or is placed in such an environment that all vital functions are suspended, as for example, exposure to cold. The waste from structural needs is inconspicuous and is usually lost sight of in discussing bacterial metabolism for it is overshadowed by the relatively prominent phenomena incidental to the fuel waste. The results of the vegetative activities are those associated with the utilization of "fuel" rather than structural materials. Purely qualitative observations upon bacterial activity, therefore, cannot be relied upon to furnish a complete survey of all the processes involved and yet the recognition of these processes is practically important wherever bacterial activity is concerned, be it in medicine, agriculture, manufacturing, or in the preparation of bacterial products. A brief résumé of certain striking instances where bacterial metabolism plays a prominent part has been published previously.¹ It is apparent that a more definite knowledge of the actual chemical reactions involved in metabolism will be advantageous both to

¹ Kendall, *Jour. Med. Research*, 1911, 25, p. 117; *Boston Med. and Surg. Jour.*, 1913, 168, p. 825.

explain the observed changes which bacteria bring about and to serve as a theoretical basis upon which to erect a rational and specific method for correcting them.

The study of bacterial metabolism, furthermore, may confidently be expected to throw a certain amount of light upon cellular metabolism in general, for it must be conceded that the metabolism of the human body reduced to its lowest terms is unicellular metabolism, modified along special lines to meet certain necessary physiological divisions of labor.

The metabolism of a certain number of bacteria has been studied chemically, using methods which have been described in detail in earlier publications.¹ The results of these experimental metabolic studies which were concerned chiefly with the utilization of carbohydrate and protein were suggestive, and fairly definite. Briefly they appear to substantiate the theory that "fermentation takes precedence over putrefaction," at least for many bacteria of interest in medicine. The question naturally arises: can these results be fairly duplicated at any time, and with any strain of the organism in question? Table 1 contains the analytical data for *B. typhosus* and *B. coli* which have been studied in the Northwestern University Medical School, using the same methods and strains of organisms as those referred to above.

This holds true only for broths made from the same type of ingredients using a uniform technic of preparation throughout. Broth made from meat extract and peptone would give consistently different results from similar broth made from meat juice and peptone; these differences furthermore become more and more marked as the protein constituents of the media become more and more simple in their structure. The substitution of amino acids for peptone would bring about an entirely new readjustment of proteolytic values. On the other hand, standard nutrient broth made in accordance with a definite formula will give fairly concordant results, provided the cultures are of maximum vegetative activity. This is shown in the table.

It is reasonable to expect that physiological limits may be determined for various groups of bacteria in terms of quantitative

¹ *Loc. cit.*

chemical data and that unusual variants which differ from their respective types shall be scrutinized from entirely new points of view. Such chemical procedures will not supplant biological

TABLE 1.

	PLAIN BROTH		DEXTROSE BROTH	
	Free NH ₃ as mgs. N ₂ per 100 c.c. Broth	Ammonia Nitrogen Total Nitrogen	Free NH ₃ as mgs. N ₂ per 100 c.c. Broth	Ammonia Nitrogen Total Nitrogen
		per cent		per cent
<i>B. coli</i> I. <i>a</i>	19.60	+5.01	+4.20	+0.53
" " <i>b</i>	16.10	+5.35	+1.40	+0.29
" " II. <i>a</i>	21.10	+6.74	+0.70	+0.29
" " <i>b</i>	21.70	+7.58	0.00	0.00
" " III. <i>a</i>	17.50	+7.80	+1.40	+0.64
" " <i>b</i>	22.40	+7.80	0.00	0.00
<i>B. typhosus</i> I. <i>a</i>	7.00	+2.89	+1.00	+0.58
" " <i>b</i>	8.40	+3.58	+0.70	+0.25
" " II. <i>a</i>	8.75	+2.60	-3.50	-1.70
" " <i>b</i>	4.90	+1.63	-0.70	-0.20
" " III. <i>a</i>	3.50	+1.68	+2.55	+1.27
" " <i>b</i>	6.00	+2.20	+1.40	+0.50
" " IV. <i>a</i>	4.55	+2.13	+2.80	+1.33
" " <i>b</i>	5.60	+2.05	+1.40	+0.50
" " V. <i>a</i>	10.00	6.65	0.00	0.00
" " <i>b</i>	10.50	3.79	+0.35	+0.13

The horizontal columns "*a*" and "*b*" indicate respectively the amounts of ammonia produced by various strains of *B. coli* and *B. typhosus* respectively in standard nutrient broth, with and without one per cent of dextrose in Boston and Chicago. Column "*a*" represents the values obtained in Boston; column "*b*" the corresponding values obtained in Chicago eighteen months later, using the same strains.

reactions such as agglutination tests for purposes of identification, but it may be confidently predicted that quantitative measurements of bacterial activity will be of material value in the ultimate reduction of this activity to a definite chemical basis.

REPORT OF A CASE IN WHICH THE FUSIFORM BACILLUS WAS ISOLATED FROM THE BLOOD STREAM.*

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In the year 1896 Vincent¹ described a fusiform bacillus associated with the spirilla which he believed to be the etiological agent of hospital gangrene. Vincent observed that the microorganisms associated with that disease were not unlike those found in certain cases of angina.

Two years previous to the work of Vincent, Plaut² reported five cases of angina caused by "Miller's bacillus," which was apparently the same organism as that described by Vincent. The organism is frequently referred to in the German literature as the Plaut-Vincent bacillus, while to the French and English writers it is known as the bacillus of Vincent or *B. fusiformis*. The earlier investigators were unsuccessful in their attempts to isolate the fusiform bacillus, and hence only certain morphological characters of the organism could be studied. Abel³ was probably the first to cultivate this organism in the laboratory. He succeeded in growing *B. fusiformis* in a mixed culture with an unidentified diplococcus; after the second transplant, however, the fusiform bacillus was lost and further attempts to cultivate the organism were unfruitful.

Vincent cultivated *B. fusiformis* in the broth of Martin. In this medium the organism grew in long filaments, leaving the broth clear. This author observed that when transplanted from broth of Martin to media containing native albumin, such as ascitic fluid or blood serum, the organism would again resume its original fusiform shape. Zuber and Veillon⁴ isolated an anaerobic organism from two cases of appendicitis which were probably identical with the bacillus of Plaut and Vincent. Lewkowitz⁵ isolated the fusiform bacillus by growing it in the deeper layers of ascitic

* Received for publication June 30, 1913.

¹ *Ann. de l'Inst. Pasteur*, 1896, 10, p. 488.

² *Deutsch. med. Wchnschr.*, 1894, 20, p. 920.

³ *Centralbl. f. Bakteriöl.*, I Orig., 1898, 24, p. 1.

⁴ *Arch. de m  d. exp  r. et d'anat. path.*, 1898, 10, p. 517.

⁵ *Bull. de l'Inst. Pasteur*, 1903, 1, p. 825.

glucose agar. Ellerman¹ was equally successful with his cultures on slant serum agar under pyrogallic acid and NaOH. Lewkowicz and Ellerman were unable to obtain cultures on any media except such as contained native albumin.

In this country *B. fusiformis* has been isolated and carefully described by Weaver and Tunncliff;² these authors cultivated an organism under anaerobic conditions which corresponded morphologically and biologically with those described by the European writers. In 1900 Harris³ isolated an anaerobic organism from a liver abscess which he christened *B. mortiferus*. The bacillus studied by Harris appears, however, to have been closely related to *B. fusiformis*.

MORPHOLOGY AND CULTURAL CHARACTERISTICS.

Preparations made from lesions containing *B. fusiformis* present a characteristic picture of long, slender, usually deep-staining rods associated with spirilla which ordinarily stain less intensely. The bacilli vary in length from 6 to 12 microns by 0.6 to 1 micron in breadth. They are spindle shaped, being swollen in the center and having distinctly pointed extremities. The bacilli stain unevenly, somewhat resembling the diphtheria bacilli in this respect. They are readily stained by any of the anilin dyes, and are gram-negative. The protoplasm of the bacilli seems to be concentrated in small masses or granules; these vary in number, according to the length of the bacillus, ranging from one to seven, the average being from two to four granules in each organism. The granules take the stain very deeply while the intervening space does not stain at all or is only very slightly tinged. Stained by the Romanowsky method the granules show distinct metachromasia.

The coexistence of the spirilla with bacilli has been the theme of much speculation; it has until quite recently been assumed that there was a symbiotic relation between two distinct microorganisms, the one being unable to thrive if the companion were absent. The cultivation of *B. fusiformis* by Abel, Vincent, Lewkowicz, Ellerman, and others would seem to disprove this theory, however, as these authors cultivated the bacillus separate from the spirillum. Tunncliff⁴ believes the spirillum represents another stage of the same organism, as she saw spirilla develop from the bacilli which were being cultivated on blood agar and ascitic agar.

The question whether or not *B. fusiformis* and the associated spirillum are motile is still an open question, if one is to judge from the literature. Bernheim⁵ and Abel state that the bacillus has a slight vacillating movement. These authors made their observations on organisms taken direct from the buccal cavity, and it is therefore conceivable that the motility was due to some of the motile spirochetes normally found in the mouth. Carnot and Fourniet⁶ and Plaut found the bacilli and spirilla to be motile when taken directly from the mouth, and the "spirochaetae" in culture retained their motility. On the other hand, Weaver, Tunncliff, Lewkowicz, and Ellerman state that *B. fusiformis* (from the cultures) is non-motile. Tunncliff later observed vibratory but no progressive motion from some of her cultures. Costa⁷

¹ *Centralbl. f. Bakteriöl.*, I Orig., 1904, 37, p. 729.

² *Jour. Infect. Dis.*, 1907, 4, p. 8; *ibid.*, 1905, 2, p. 446.

³ *Jour. Exper. Med.*, 1901, 6, p. 519.

⁴ *Jour. Infect. Dis.*, 1911, 8, p. 316.

⁵ *Centralbl. f. Bakteriöl.*, I Orig., 1898, 23, p. 177.

⁶ *Compt. rend. Soc. de biol.*, 1901, 53, p. 143.

⁷ *Ibid.*, 1909, 67, p. 866.

examined pus containing *B. fusiformis* and found they were motile, provided they were not exposed to the air, but lost their motility upon coming under the influence of oxygen. The evidence is that no definite motility has been observed from cultures. Graupner¹ and Plaut and Veszprémi² have, by a special staining method, been able to demonstrate *B. fusiformis* to possess numerous peritrichic flagella. The presence of such flagella would naturally suggest that the organism is motile. Whether or not the organisms studied by these investigators were the same as those described by other writers must at present remain an open question.

The fusiform bacillus is a non-spore-bearer and does not form capsules. The organism under consideration is regarded by most authors as an obligate anaerobe. It will grow under aerobic conditions, however, after it has been cultured anaerobically for some generations, as the work of Weaver and Tunncliff indicates. Abel, Vincent, Carnot, and Fourniet cultivated the organism aerobically in mixed cultures. When the organism is planted upon media containing native albumin and placed in oxygen-free environment, there appear small delicate colonies resembling those of the pneumococcus in from one to five days' incubation at 37° C. The colonies attain a size of from 1-2 mm. in diameter. After a few days' incubation the cultures are said to emit a foul, disagreeable odor. On glucose serum-agar Lewkowicz obtained a continuous undulate growth of a grayish hue. On ordinary slant agar Zuber and Veillon, and Weaver and Tunncliff obtained a delicate growth, which, according to the latter authors, appeared along the line of inoculation, resembling a cloud on the surface of the medium. The development in gelatin at room temperature is very slow, and there is no liquefaction. Neither acid nor gas is formed on sugar media. In serum broth the organism grows in long filaments which settle, leaving a perfectly clear fluid.

PATHOGENICITY.

The fusiform bacillus is a normal inhabitant of the mouth (Mühlens,³ Hartman, Miller⁴) and it has also been found on normal genitalia. In pathological conditions it has been found associated with a variety of necrotic lesions, such as ulceromembranous angina (Plaut, Vincent, and Bernheim) and ulcerative stomatitis (Perthes,⁵ Ellerman, and others).

Vincent, Coyoyn,⁶ and others have found it to be the cause of hospital gangrene. Veillon and Zuber observed that the organism may be a cause of appendicitis. It has likewise been found in gangrenous laryngitis (Bernheim and Pospischill⁷), empyema of the antrum of Highmore and fetid bronchitis (Silberschmidt⁸) associated with a metastatic abscess of the femur. Weaver, Tunncliff, and others have found this organism in cases of noma. Quite recently Bevacqua⁹ reports a case of pseudoelephantiasis in which he was able to demonstrate typical fusiform bacilli. Ellerman¹⁰

¹ *München. med. Wchnschr.*, 1902, 49, p. 727.

² *Centralbl. f. Bakteriöl.*, 1907, 44, pp. 332, 408, 515, 648; *ibid.*, 45, p. 15.

³ *Ztschr. f. Hyg. u. Infektionskrankh.*, 1906, 55, p. 81.

⁴ *Die Mik. d. Mundhöhle*, Leipzig, 1892.

⁵ *Arch. f. klin. Chir.*, 1899, 59, p. 111.

⁶ *Ann. de l'Inst. Pasteur*, 1895, 9, p. 660.

⁷ *Jahr. f. Kinderh.*, 1897, 46, p. 434.

⁸ *Centralbl. f. Bakteriöl.*, 1901, 30, p. 159.

⁹ *Ibid.*, 1913, 68, p. 182.

¹⁰ *Ztschr. f. Hyg. u. Infektionskrankh.*, 1907, 56, p. 453.

found *B. fusiformis* in the throats of 22 out of 38 cases of scarlet fever examined. Costa found the organism in a case of abscess of the kidney. Corbus¹ reports a number of cases of gangrenous balanitis probably due to this same organism.

In the preantiseptic days, cases of hospital gangrene were very common. Percy in a report issued from l'Hôtel-Dieu, Paris, states that 98 per cent of the wounds treated at that hospital were infected with the organism of hospital gangrene (cited by Vincent). During the European wars in the early part of the eighteenth century, hospital gangrene caused a very large mortality among the wounded. It was not uncommon for the surgeons attending such cases to become infected.

It is apparently very difficult, however, to produce an experimental infection. Vincent inoculated himself with the organisms which resulted only in an insignificant pustule. Gemy inoculated three persons with material from severe cases of hospital gangrene without success. It is to be assumed, therefore, that certain conditions must be fulfilled before an infection can take place. It is probable that the serum of a normal individual contains antibodies in the form of either lysins or opsonins by virtue of which the organisms are immediately destroyed. Inoculation experiments practiced upon animals have likewise met with but little success. Some investigators report that their animals succumbed with symptoms of toxemia a few hours after inoculation of large doses was made. Vincent produced a subcutaneous abscess in a cachectic rabbit by inoculating it with material from a patient suffering from hospital gangrene. The abscess thus produced evacuated itself spontaneously, on the sixth day following the inoculation, of a quantity of fetid pus containing the typical organisms. The same experiments performed upon healthy animals were negative. Silberschmidt produced abscesses in mice into which he had inoculated pus from a femoral abscess. The abscesses thus produced contained the fusiform bacilli in large numbers. Costa inoculated a guinea-pig with pus from a kidney abscess containing the organisms and thus succeeded in producing an abscess in the animal after an incubation period of several weeks.

In reviewing the work of the various investigators who have attempted to produce experimental lesions, one is struck by the

¹ *Jour. Am. Med. Assn.*, 1913, 60, p. 1769.

fact that the successful inoculations have almost invariably been made with material taken directly from the patient. Veszprémi, however, succeeded in producing lesions in animals by inoculating these with pure cultures. He invariably found other organisms associated with the fusiform bacillus in the lesions produced, and it is therefore difficult to determine which organism was responsible for the pathological changes.

By inoculating an animal with a quantity of pus containing the organisms it is not improbable that the ferments liberated by the disintegrating leukocytes produce local changes which favor the development of the infectious agent, either by virtue of the biochemical changes produced or by protecting the organisms from the influences of the antibodies. However, the possibility of the organism rapidly losing its virulence when cultivated artificially should not be overlooked.

In the light of Bail's¹ aggressin theory, the explanation would be very simple. According to this author, pathogenic bacteria have the property of forming substances which protect them from the action of the antibodies. He found, for instance, that pus from a streptococcic abscess, when heated sufficiently to destroy the bacteria, would produce no lesion if injected into an animal. If, however, an avirulent streptococcus was injected together with such material, the otherwise harmless organism became pathogenic. More recent investigations have shown that this same "aggressin action" may be obtained by the injection of bacterial filtrates. Further investigation of this problem is necessary, however, before a satisfactory explanation of the phenomenon can be offered.

CASE REPORT.

E. N. H., male, age 37, ticket collector at a theater, entered the Swedish Hospital, Minneapolis, September 30, 1912, on the service of Dr. S. P. Rees, giving the following history:

Was at work and apparently well up to three weeks previous to his admission, when he was taken with a sudden chill. After that he had to stop work occasionally because of a feeling of general malaise, altho he had no definite symptoms. He stated that his teeth were in very bad condition and that he suffered from night-sweats the week before entering the hospital.

¹ *Arch. f. Hyg.*, 1905, 52, p. 272; *Deutsch. med. Wchnschr.*, 1905, 31, p. 1788.

Physical examination revealed nothing abnormal with the exception of a hectic flush on cheeks, and a very severe pyorrhea alveolaris accompanied by an extremely foul breath. The condition in the mouth did not yield to ordinary antiseptic treatment, and a closer examination revealed extensive ulceration and necrosis of the upper jaw.

During the stay in the hospital, patient ran a septic temperature with remissions in the morning to 98° F. and exacerbations in the afternoon to 104°. The pulse ranged between 100 and 135, but always regular; respiration 20 to 25.

Examination of the urine was negative except for a trace of albumin.

Leukocyte counts were as follows: October 2, 5,800; October 6, 2,400; October 12, 2,600.

Widal reaction on October 8 was negative, and Wassermann also negative.

Repeated examinations of the sputum did not reveal any tubercle bacilli. The laboratory reports of October 6 and 10 stated that diphtheria-like bacilli were present in the sputum. Subsequent more careful examinations revealed numerous spirilla and fusiform bacilli in the direct smears taken from the ulcerations in the mouth, and this led to a diagnosis of ulceration and gangrene due to infection with the fusiform bacillus.

Patient was in the hospital 28 days. At no time did he show any signs of any great discomfort or of pain. He developed no special symptoms up to a few days before his death, when he slowly sank into a stupor. Two days before his death, a blood culture was taken, the results of which will be described presently. Patient died on October 27, 1912, about seven weeks after the onset of the disease.

Abstract of autopsy protocol.—Body is well developed, but extremely emaciated. Embalming fluid is present in the body cavities. The skin around the nostrils and upper lip shows greenish discoloration. On reflecting the lips, the bone of the superior maxilla is blackened and necrotic. Probing shows destruction of the bone as far as the antrum of Highmore on the left side, and extending into the nasal cavities. The soft palate is necrotic, and the hard palate is rough and granular. There is a foul odor present, but no evidence of abscesses or gumma. The spleen is enormously increased in size, measuring $19 \times 10 \times 5$ cm.

The findings of the other organs have no special import.

Blood culture.—The blood culture, made by aspirating the basilic vein 48 hours prior to exitus, was taken on plain agar, ascitic agar, and Loeffler's blood serum. The tubes were incubated for four days at 37° C. but no growth appeared. They were then incubated anaerobically in a Novy jar under hydrogen at 37° C. and after 48 hours the tubes of ascitic agar showed a scanty, rather dull, translucent, continuous growth. A few denser colonies, 1-2 mm., were scattered over the surface. The other media showed no growth.

Smears from these cultures showed a pure growth of fusiform bacilli which did not stain by Gram's method. Staining with Giemsa's stain diluted 1:10 for from 2 to 24 hours brought out one to two deeply staining bodies of a purplish-red color in each bacillus. The bacilli were distinctly canoe-shaped, being broadest at the middle and tapering to a point at both ends. Some were joined end-to-end in pairs, as if in the process of division. Generally, only one large granule was present in each of the paired bacilli, while most of the single bacilli contained two smaller granules. The organisms from the original culture were fairly uniform in size and shape. Some preparations showed small clumps of bacilli arranged radially about a central point.

In a general way, the morphology and staining of the bacilli corresponded almost exactly with the pure cultures described by Weaver and Tunnicliff.

Subcultures were planted on plain agar, ascitic broth, plain broth, ascitic agar, and Loeffler's blood serum. Tubes of each were incubated aerobically as well as anaerobically. At this time, no growth was obtained on the aerobic cultures. The anaerobic cultures showed growth on all but the plain agar; the best growth appeared on the ascitic agar.



FIG. 1.—*A*, from an early culture on ascitic agar; *B*, from a blood agar culture; *C*, from an early culture; *D*, from blood agar showing spiral forms; *E*, some of the various forms the organism may assume in old cultures.

It was only after a number of anaerobic subcultures that we succeeded in growing the bacillus aerobically, and finally upon media containing no native albumins. But after this was ultimately accomplished, the cultures showed no less development under aerobic than under anaerobic conditions.

The morphology of the organisms from the original cultures and first transplantations was, as we have already said, fairly uniform. But with the subsequent

transplants marked polymorphism developed. This, just as Tunncliff found, was especially noticeable when the transplantations were made on a medium upon which they had not previously grown. In the case of horse-blood agar, the organism showed a marked tendency to form spirals and long filaments. Some of these long filaments attained a length up to 100 microns and contained as many as 20 or 30 granules.

The relation between the fusiform bacilli and the spirilla usually found associated with them in direct smears is still an open question. The early writers, especially, deny that they are kin to one another. However, the later writers (Tunncliff, Perthes, Neuof, and others) believe them to be different forms of the same organism. Our work would seem to confirm fully the opinions of these later authors. When the cultures from horse-blood agar, showing the spiral forms, were transplanted on ascitic agar, the transplants would show only the fusiform bacillus. Replanted on horse-blood agar, the spirals would often reappear.

Animal inoculations.—Rabbits, guinea-pigs, mice, and rats were inoculated intravenously, subcutaneously, intraperitoneally, intratesticularly, and into the anterior chamber of the eye, but invariably with negative results.

Viability.—The viability of the different strains described in the literature shows marked variation. Veszprémi and others were unable to cultivate the organism for more than two generations. Ellerman cultivated the organism for nine generations without noticing any appreciable loss in vitality. Our strain was successively transplanted for about 20 generations and the viability was fully retained. The cultures at the end of this time contained many involution forms, however. Several cultures on ascitic and on blood agar were kept at room temperature in paraffin-sealed tubes for a period of about six months. At the end of that time, transplants were made which were placed under aerobic as well as anaerobic conditions. Growth occurred only on the anaerobic cultures. From these we again succeeded in cultivating the organism aerobically.

Aside from the fact that our strain could be adapted, through successive transplants, to grow on media not containing native albumins, and under aerobic as well as under anaerobic conditions, the bacillus here described seems to be identical, culturally and

morphologically, with the fusiform bacillus described by other writers.

As far as we know, this is the first case on record in which the fusiform bacillus has been isolated from the blood stream. We therefore believed it to be of sufficient interest to warrant the presentation of this paper.

We wish to express our thanks to Dr. S. P. Rees for furnishing us the opportunity to study this case and for the history and hospital records.

FUSIFORM BACILLI: CULTURAL CHARACTERISTICS.*

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In a previous communication we reported a simple method for isolating fusiform bacilli, and methods for cultivation without the use of complex anaerobic apparatus. The strains thus isolated have come from varied sources and have been studied to determine whether they could be differentiated, one from the other, according to their cultural characteristics, and whether, if differentiation is possible, the grouping of the cultures has any relation to their source.

Altho there is an extensive literature on the cultural characteristics of the fusiform bacillus, most of the reports deal with one or at most a few strains and are of little value for comparison and differentiation of strains. For this reason we shall report simply our own results.

CULTURES STUDIED AND THEIR SOURCES.

1. Noma—recovering after salvarsan.
2. Ulceration of tongue from carious tooth.
3. Vincent's angina.
4. Discharging ear.
5. Discharging ear.
6. Pyorrhea.
7. Spongy bleeding gums.
8. Vincent's angina.
9. Vincent's angina.
10. Pyorrhea.
11. Vincent's angina.
12. Noma.
13. Carious tooth.
14. Vincent's angina.
15. Discharging ear.

Morphology.—The typical organism from the lesion is a double-pointed bacillus, containing one or more granules. The morphology is very variable in cultures, varying with the culture medium employed. On or in solid media the morphology is similar, tho the forms are somewhat longer. In the water of condensation of agar

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slants or in fluid media there is a tendency toward the development of filamentous forms, often forming tangled threadlike masses. In some of the filaments the granules may be absent. The typical organism is straight or somewhat bent. The filaments are at times sinuous and where granules are absent simulate a loosely wound spirochete. These are more common in fixed preparations. In fluid media, on or in solid media, when grown under identical conditions our strains showed no morphological characteristics that would be of value in differentiation of one strain from another. The only variation noted was the tendency of Nos. 2 and 3 to produce a greater proportion of short forms.

Motility.—Repeated examinations, in hanging-drop preparations and with dark field illumination, of original material and of bacilli from the most varied types of culture media, have failed to show any evidence of motility.

Gram stain.—None of the strains retained the dye.

Colony morphology.—The characteristic colony, as already described, develops only in the depths of agar.

General characteristics of growth.—As we have stated, serum or blood is necessary for satisfactory cultivation of the strains. In our previous article we referred to the use of a semi-solid agar-gelatin mixture which facilitated the transfer of stab cultures. We have found that one-half of 1 per cent agar with the addition of one-third of its volume of serum gives satisfactory results and is much easier to prepare. After isolation, growth may take place in serum-free media for several generations, but for continued vigorous development and surety of transfer a serum medium has been found necessary.

On or in agar, the growth of all the strains was the same. In stab or shake culture a puff-ball appearance is present, as one would expect from the appearance of the colony. On serum or blood agar the growth is less characteristic, consisting of a delicate greyish-white non-adherent layer becoming more opaque with longer incubation and developing a heaped-up appearance.

In broth a flocculent growth occurs at the bottom of the tube, leaving the upper portion clear. The simplest method for cultivation in serum broth is to sterilize the broth with a layer of albolene and add to each tube a small amount of heated serum.

Indol production.—All strains produced a large amount of indol.

Odor.—All cultures had a similar disagreeable odor.

Viability.—After cultivation for several generations the strains remain viable for a long period. Some strains were viable 67 days when preserved on ice. In the incubator three of four strains were viable up to six months.

The viability of freshly isolated strains was not tested. Although various batches of media are unequally adapted for their cultivation, they can be easily kept alive in semi-solid serum media. Some of our strains are now in the fiftieth to sixtieth generation.

SUGAR FERMENTATIONS.

No.	Dextrose	Galactose	Levulose	Arabinose	Raffinose	Lactose	Saccharose	Dextrin	Maltose	Mannite	Mannose	Dulcite	Rhamnose	Inulin	Glycerin
1.....	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
2.....	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
3.....	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
4.....	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
5.....	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
6.....	++	++	++	-	-	-	++	-	-	-	-	-	-	-	-
7.....	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
8.....	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
9.....	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
10.....	++	++	++	-	-	-	+	-	-	-	-	-	-	-	-
11.....	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
12.....	++	++	++	-	-	-	++	-	-	-	-	-	-	-	-
13.....	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
14.....	++	++	++	-	-	-	+	-	-	-	-	-	-	-	-
15.....	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

No gas formation was noted in any of the tubes. This fact, as well as the differences in morphology, differentiates the fusiform bacillus from the bacillus necrosis which produces gas from dextrose.

Method.—To a sugar-free semi-solid agar sufficient sugar solution was added to bring the content to 1 per cent, and finally the necessary serum. Litmus was used as an indicator, the color being restored by softening the agar and pouring into a petri-dish. Nearly all the results were later checked by titration which gives sharper readings. For sterilization the sugar solutions were usually heated 10 minutes. In the case of maltose, sterilization was accomplished by filtration. In a few series the inoculated tubes showed a slight increase of acidity over the controls. As the non-fermenting controls gave the same change, this was referable to

slight cleavage of the sugar and the formation of fermentable monosaccharides. This deduction was further proved by the fact that the positive controls gave a marked increase of acidity comparable with the acid production of some fusiform strains with saccharose.

Relation of the fusiform bacillus to the spirochetes from the same source.—In the smears made from the original material, loosely wound spirochetal forms comparable with those found in fusiform cultures are present. The typical spirochetal forms seen in the original material have never been encountered in pure cultures of the bacillus. The cultural forms simulating spirochetes have been found in fixed preparation. Repeated examinations with dark field illumination have revealed no forms that could in any way be compared with those found in original material. Furthermore the spirochetes in the original material, morphologically at least, may be of two or possibly three varieties. These can be kept alive in mixed culture in coagulated horse serum, the fusiform bacilli dying out on transfer. When pure cultures of fusiform bacilli are planted in this medium no change of morphology takes place. The motility of the spirochete and the lack of motility of the fusiform bacillus form the strongest evidence against their identity. The observations of cultural forms have embraced the growth on the most varied media and at every stage of incubation.

SUMMARY.

The fusiform bacilli isolated by us fall in two groups, one group fermenting saccharose, the other not.

There is no relation between these groups and the source of the culture.

The fusiform bacillus and the spirochetes accompanying it are separate and distinct organisms.

THE DIFFERENTIATION OF FECAL STREPTOCOCCI BY THEIR FERMENTATIVE REACTIONS IN CARBOHYDRATE MEDIA.*†

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The use of fermentative reactions of the streptococci in carbohydrate media for tracing out the systematic relationships within this group of organisms was first suggested by Gordon¹ in 1904. His suggestions were further worked out and elaborated by Houston² in the following year and by Andrewes and Horder³ in 1906. The two last-named observers made a careful study of the records of the cultures which had been isolated by Gordon and Houston, and of a number of strains isolated by themselves from a large variety of sources. From the results of their observations they divided the group streptococci into seven sub-groups or species which they considered to be the main types or type centers about which the more variable forms were clustered. The first of these types fermented saccharose, salicin, and coniferin, and is referred to by them as *Str. equinus*. The second type, for which they proposed the name, *Str. mitis*, fermented saccharose, lactose, and salicin. The third type, *Str. pyogenes*, also fermented saccharose, lactose, and salicin but exhibited some minor variations of morphology and pathogenic properties. The fourth type, *Str. salivarius*, fermented saccharose, lactose, and raffinose. The fifth type, *Str. anginosus*, fermented saccharose and lactose. The sixth type, *Str. fecalis*, fermented saccharose, lactose, salicin, coniferin, and mannite. The seventh type, which corresponded to the pneumococcus, fermented saccharose, lactose, raffinose, and inulin. Andrewes and Horder also pointed out the scarcity of lactose fermenting strep-

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¹ *Rpt. of Med. Off. to Local Gov't Bd. Great Britain*, 1902-3, 32, p. 421; *ibid.*, 1903-4, 33, p. 38.

² *Ibid.*, 1904-5, 34, p. 326; *Rpt. to London County Council*, July 11, 1905.

³ *Lancet*, 1906, 171, p. 708.

tococci in horse dung and street air. They found, however, that most of the strains isolated from human feces acted on this sugar. In the same year Houston showed that lactose fermenting strains were very numerous in cow dung but that mannite was not fermented by bovine types while 24 per cent of the human strains attacked this carbohydrate. Raffinose was fermented by the majority of bovine streptococci while but 32 per cent of the human strains fermented this sugar.

In 1910 Winslow and Palmer¹ suggested that these fermentative reactions of the fecal streptococci on various carbohydrates might be used as a means for differentiating between cultures derived from human and animal excreta. The importance to water analysts of a test of this nature can scarcely be overestimated since by this means it would be possible to differentiate between pollution of human and animal origin in water supplies. Winslow and Palmer carefully reviewed the work of the English observers and presented a study of 302 cultures of streptococci isolated by them from human, equine, and bovine feces. These cultures were grown in broth containing dextrose, lactose, saccharose, mannite, and raffinose, and the acidity produced by the fermentation of these different carbohydrates determined by titration. The data accumulated in these experiments were interpreted by the statistical or biometrical method which has been so ably applied to the systematic study of the family *Coccaceae* by the Winslows. The basis of this system rests upon the "study of the frequency with which certain characters or combinations of characters occur. The type centers 'or species' are defined by the occurrence of a large number of individuals with a given characteristic. These common types among such variable organisms as the bacteria may properly be considered as representing species about which the rarer varieties are grouped."

These observers conclude that the "presence of streptococci forming over 3.5 per cent of acid in dextrose broth would seem in general to be characteristic of human stools. Second, that raffinose fermenting forms appear to be more abundant in bovine than in human feces. Third, and of most importance, that mannite

¹ *Jour. Infect. Dis.*, 1910, 7, p. 1.

fermenting streptococci, which make up about one quarter of the human streptococci, are very rare in the feces of the cow and horse."

The report upon 100 additional strains from human feces and 71 from water was published by Houston¹ in 1910. In the same year Gordon² also reported the results of his investigation upon streptococci isolated from diseased throats. In 1912 there appeared several papers dealing with the systematic relationships of these organisms as differentiated by their fermentative powers in various carbohydrate media. Broadhurst³ reported upon 100 strains isolated from milk and Hilliard⁴ upon 65 strains from normal and diseased throats.

These papers and the earlier literature bearing upon the subject have been carefully examined and reviewed by Winslow⁵ in a paper published the same year. This observer compared the relative merits of the methods used by the English and American workers, and, after an exceedingly careful analysis of the data published, has endeavored to formulate a rational and clear interpretation of the, up to that time, somewhat confused mass of facts. In general the conclusions of the American and English workers are in close agreement. Winslow has pointed out, however, that, on the whole, the work of the English observers has been somewhat less accurate than that of the Americans on account of the cruder methods of technic used. The English used litmus as an indicator of acid production. They added a few drops of litmus solution to their nutrient carbohydrate broth and determined the positive or negative fermentation simply by the change in color of the litmus media. Winslow and the other American observers have emphasized the importance of a quantitative acid determination and in their work carefully titrated their cultures against an N/20 sodium hydrate solution using phenolphthalein as an indicator. Winslow is inclined to attribute any discrepancy between the results of the English and American observers to the different methods of technic employed. For a detailed review of the entire literature on the subject the reader is referred to this excellent paper by Winslow.

¹ *Fifth Rpt. on Research*, Metropolitan Water Bd., London, 1910.

² *Rpt. Med. Off. to Local Gov't Bd., Great Britain*, 1910-11, 40, p. 302.

³ *Jour. Infect. Dis.*, 1912, 10, p. 272.

⁴ *Amer. Jour. Dis. of Children*, 1912, 3, p. 287.

⁵ *Jour. Infect. Dis.*, 1912, 10, p. 253.

TABLE 1.

SHOWING THE ACIDITIES PRODUCED BY STREPTOCOCCI ISOLATED FROM HUMAN FECES IN DEXTROSE, LACTOSE, SACCHAROSE, MANNITE, AND RAFFINOSE BROTH.

Culture	Dextrose	Lactose	Saccharose	Mannite	Raffinose
1.....	3.4	2.3	2.1	1.3	— .5
2.....	3.7	2.3	1.9	1.4	— .5
3.....	3.6	2.5	1.4	1.6	— .5
4.....	3.8	2.3	2.3	1.4	— .5
5.....	3.9	2.4	2.7	1.5	— .5
6.....	3.9	2.2	1.8	1.6	— .5
7.....	3.8	2.5	2.1	1.6	— .5
8.....	3.8	2.6	2.2	1.5	— .5
9.....	2.9	1.5	— .5	— .5	— .5
10.....	2.6	0.1	— .3	— .5	— .5
11.....	2.3	1.1	— .4	— .5	— .5
12.....	5.1	2.5	— .5	— .4	— .5
13.....	3.0	2.3	2.4	— .5	— .2
14.....	2.9	2.3	2.5	— .5	— .5
15.....	3.5	— .2	— .5	2.4	— .5
16.....	0.5	— .2	— .5	— .5	— .5
17.....	0.2	0.1	— .5	— .5	— .5
18.....	1.9	— .1	— .5	— .5	— .5
19.....	2.6	2.2	2.6	— .5	— .5
20.....	3.4	2.1	— .5	— .5	— .5
21.....	3.4	2.4	— .5	1.4	— .1
22.....	3.1	2.5	2.0	1.4	— .2
23.....	3.3	2.3	1.8	1.4	0
24.....	3.2	2.4	1.9	1.2	— .2
25.....	3.4	2.2	1.7	1.4	0.3
26.....	3.2	1.4	1.8	1.4	0.3
27.....	3.1	2.3	2.2	1.5	— .2
28.....	3.7	2.1	2.2	1.5	— .5
29.....	3.6	2.4	2.1	1.6	0
30.....	3.6	2.5	2.3	1.6	0
31.....	3.3	2.4	2.1	1.5	.1
32.....	3.2	2.3	1.8	1.4	— .5
33.....	3.6	2.4	2.1	1.6	— .1
34.....	3.6	2.5	2.2	1.5	0.1
35.....	3.5	2.4	1.7	1.5	0.5
36.....	3.4	2.2	2.3	1.4	0.2
37.....	3.3	2.2	2.0	1.5	0
38.....	3.2	2.4	2.0	1.5	— .4
39.....	3.4	2.4	1.9	1.4	0.5
40.....	3.4	2.3	1.3	1.5	— .2
41.....	2.5	1.8	2.1	1.4	— .5
42.....	2.6	1.9	1.6	0.9	— .2
43.....	2.2	1.8	1.4	0.9	— .5
44.....	2.6	1.8	1.4	1.1	— .5
45.....	2.3	1.8	1.6	1.2	0.2
46.....	2.6	1.9	1.4	1.2	0.2
47.....	3.0	1.9	1.3	1.1	0.2
48.....	2.9	1.7	1.4	1.0	0.3
49.....	2.2	1.7	1.8	1.7	— .5
50.....	3.6	2.6	2.2	1.6	0.2
51.....	3.6	1.8	2.0	1.6	0.1
52.....	3.5	2.7	2.1	1.6	0.3
53.....	3.6	2.8	2.2	1.6	0.2
54.....	3.4	2.7	1.6	1.6	— .3
55.....	3.4	2.6	2.1	1.6	0.8
56.....	3.3	2.3	1.8	1.5	0.5
57.....	3.3	2.7	1.7	1.3	— .1
58.....	2.4	2.5	1.8	1.2	— .4
59.....	3.2	2.4	1.7	1.3	— .4
60.....	3.3	2.5	1.9	1.5	— .2
61.....	2.7	1.6	1.4	1.1	— .3
62.....	2.5	1.4	1.3	1.1	0.4
63.....	2.6	1.5	1.4	1.0	0.3
64.....	2.5	1.8	1.5	1.2	0.4
65.....	2.7	1.5	1.6	1.2	0.2
66.....	1.8	1.5	1.0	1.1	0.4
67.....	3.5	2.0	2.1	1.4	0.7
68.....	3.5	1.6	1.7	1.3	0.2
69.....	3.8	1.7	1.6	0.9	0.4
70.....	3.2	1.8	2.1	1.5	0.7

TABLE 1—Continued.

Culture	Dextrose	Lactose	Saccharose	Mannite	Raffinose
71.....	3.5	1.8	1.8	1.3	0.6
72.....	3.6	1.8	1.7	1.3	0.3
73.....	3.8	1.8	1.6	1.3	0.5
74.....	3.5	1.9	1.7	1.3	0.3
75.....	3.7	1.8	1.6	1.1	0.5
76.....	3.6	1.8	1.7	1.1	0.4
77.....	3.7	1.8	1.5	1.3	0.5
78.....	4.0	1.9	1.5	1.3	0.4
79.....	3.8	1.8	1.7	1.3	0.6
80.....	3.7	1.8	1.8	1.3	0.4
81.....	3.9	1.7	1.7	1.2	0.4
82.....	3.4	1.8	1.8	1.3	0.5
83.....	3.8	1.9	2.0	1.4	0.2
84.....	3.6	1.8	1.5	1.4	0.5
85.....	3.6	2.2	1.8	1.2	0.5
86.....	3.8	1.8	1.8	1.3	0.7
87.....	3.6	2.1	1.8	1.1	0.7
88.....	3.5	1.8	1.6	1.2	0.6
89.....	3.6	2.0	1.9	1.2	0.6
90.....	3.7	1.7	1.7	1.3	0.1
91.....	3.6	1.8	1.7	1.3	0.9
92.....	3.7	1.8	1.9	1.1	0.5
93.....	3.5	1.8	2.0	1.5	0.9
94.....	3.7	1.9	1.6	1.4	0.7
95.....	3.9	2.2	1.7	1.4	0.5
96.....	3.6	1.8	1.3	1.5	0.5
97.....	3.5	1.9	2.3	1.5	0.5
98.....	3.4	2.0	1.7	1.5	0.5
99.....	3.6	2.2	1.9	1.5	0.6
100.....	3.8	2.2	1.5	1.3	0.6
101.....	3.2	3.2	1.6	1.3	0.6
102.....	3.9	2.6	1.8	1.4	0.6
103.....	3.0	1.9	1.8	1.2	0.2
104.....	3.7	2.1	1.5	1.5	— .5
105.....	3.5	2.2	1.9	1.2	0.6
106.....	4.7	2.1	1.6	1.5	0.4
107.....	2.9	2.2	1.7	1.4	0.5
108.....	3.2	1.8	1.8	1.1	0.2
109.....	3.8	2.1	1.7	1.3	1.4
110.....	3.9	2.4	2.1	1.8	0.3
111.....	3.5	1.5	1.5	1.5	0.6
112.....	3.0	2.1	1.7	2.1	0.5
113.....	3.7	2.9	1.7	1.3	0.4
114.....	3.0	2.1	0.8	1.3	0.4
115.....	2.9	2.8	1.7	1.4	0.5
116.....	2.7	2.0	1.9	1.1	0.3
117.....	3.5	2.4	1.6	1.1	0.4
118.....	3.4	2.4	1.5	1.4	0.6
119.....	3.7	2.7	2.4	1.7	0.6
120.....	3.8	1.7	1.7	1.3	1.0
121.....	4.0	2.2	1.7	1.5	0.5
122.....	3.9	2.0	5.3	1.1	0.4
123.....	1.8	0	0	0	0

Since the appearance of Winslow's paper two others have been published in this country, one by Bergey,¹ who regards the fermentative action of the streptococci on carbohydrates as too variable a quantity to be of value in the differentiation of these forms. He has reported entirely negative results. A second paper by Hilliard,² published in 1913, gives the results of the examination of 240 strains of streptococci isolated from normal and diseased

¹ *Jour. Med. Research*, 1912, 27, p. 67.

² *Jour. Infect. Dis.*, 1913, 12, p. 144.

TABLE 2.

SHOWING THE ACIDITY PRODUCED BY STREPTOCOCCI ISOLATED FROM HORSE DUNG IN DEXTROSE, LACTOSE, SACCHAROSE, MANNITE, RAFFINOSE, INULIN, AND SALICIN BROTH.

Culture	Dextrose	Lactose	Saccharose	Mannite	Raffinose	Inulin	Salicin
1.....	2.3	2.7	3.0	0.3	0.4	0.2	2.1
2.....	4.0	2.1	1.9	1.4	0.7	0.3	2.7
3.....	3.2	3.0	1.0	2.1	0.6	0.4	2.6
4.....	4.2	3.0	2.2	2.2	0.6	0.4	2.5
5.....	3.6	3.0	3.2	1.6	0.8	0.2	2.2
6.....	1.7	2.3	1.6	0.1	0.5	2.1	0.6
7.....	3.9	1.6	3.4	0.3	1.4	0.3	3.4
8.....	4.1	0.9	3.3	0.3	0.3	0.1	2.8
9.....	3.6	2.5	2.9	1.1	0.3	0.1	2.8
10.....	3.6	2.3	3.5	0.1	0.3	0	2.0
11.....	4.6	1.0	3.1	— .5	0.8	— .3	2.6
12.....	1.6	0.4	1.0	0.2	0.4	2.1	0.5
13.....	1.4	0.4	1.7	0.4	0.7	2.0	0.7
14.....	1.5	0.8	1.8	0.3	0.5	— .5	1.9
15.....	1.7	0.7	1.2	0.4	0.1	0.1	1.2
16.....	1.3	0.5	0.9	0.5	0.6	0.1	1.2
17.....	1.5	0.7	1.3	0.4	0.6	1.6	1.2
18.....	1.5	1.3	2.8	0.3	0.3	2.9	1.7
19.....	1.4	0.8	1.2	0.4	1.2	0.9	1.5
20.....	1.3	0.4	1.5	0.1	1.0	4.0	1.4
21.....	1.7	1.0	0.1	0.5	0.7	0.1	0.5
22.....	1.5	0.6	1.2	0.4	1.2	0.3	1.1
23.....	1.3	0.6	0.8	0.4	0.9	0.3	1.2
24.....	1.7	0.4	0.9	0.5	0.4	0.3	1.3
25.....	2.7	0.5	2.3	0.1	0.5	0.2	2.2
26.....	1.1	0.3	1.2	0.3	0.5	0	0.1
27.....	2.7	1.3	3.1	1.3	2.5	3.7	1.6
28.....	1.2	0.3	0.9	0.2	0.3	0.3	0.8
29.....	2.5	2.0	2.7	0.4	2.7	3.0	2.5
30.....	2.3	0.9	2.1	0.2	2.4	3.1	2.8
31.....	1.3	0.2	0.5	0.1	0.2	0.1	1.1
32.....	1.9	1.4	2.1	0.4	1.7	3.2	2.2
33.....	1.4	1.3	0.8	0.2	— .7	1.0	1.1
34.....	2.2	3.3	2.7	1.0	2.2	2.9	2.6
35.....	0.6	1.2	1.0	0.5	0.7	0.5	— .1
36.....	1.5	0.3	1.0	0.5	0.5	0.1	0.9
37.....	2.2	1.5	1.0	0	0.9	— .1	1.3
38.....	2.6	0.5	0.6	0	0.9	1.6	1.6
39.....	3.4	0.2	0.6	0.3	0.6	1.1	0.6
40.....	1.5	0.2	0.6	0.5	0.9	2.2	0.6
41.....	1.4	0.4	1.2	0.3	0.7	1.3	1.2
42.....	1.2	1.2	1.2	0	0.8	0.0	1.3
43.....	1.5	0.4	1.4	0.0	0.5	0.5	1.4
44.....	5.3	— .4	2.3	0.0	2.4	0.6	2.4
45.....	1.6	0.3	1.5	0.3	0.7	2.0	1.4
46.....	1.6	0.5	1.4	— .3	1.0	0.2	1.9
47.....	1.6	0.2	1.7	0.0	0.6	2.0	1.3
48.....	1.4	0.5	1.8	0.3	0.4	1.5	2.0
49.....	1.3	1.8	1.2	0.1	1.6	1.5	1.7
50.....	1.9	0.5	1.1	0.0	1.9	0.3	1.0
51.....	1.5	0.3	1.4	0.0	0.7	1.3	1.4
52.....	2.0	1.0	1.3	0.0	0.8	0.9	1.3
53.....	1.5	0.5	1.3	0.0	1.1	1.8	1.4
54.....	1.3	1.8	1.4	0.3	1.3	1.8	1.2
55.....	1.6	0.2	1.5	0.0	0.8	1.4	0.9
56.....	1.8	0.8	1.3	0.0	0.4	1.0	1.1
57.....	1.3	1.9	2.0	— .2	1.5	1.1	1.9
58.....	1.2	0.2	1.2	— .4	1.0	1.0	1.3
59.....	1.5	0.6	1.2	0.7	1.0	0.0	1.4
60.....	1.5	0.7	1.4	— .4	0.5	2.5	1.4
61.....	1.5	0.5	1.6	0.0	0.6	1.2	1.2
62.....	1.7	1.3	1.2	0.0	0.9	1.2	1.3
63.....	1.2	0.5	0.9	0.0	0.5	0.0	1.0
64.....	1.3	0.7	1.5	0.0	0.5	1.3	1.1
65.....	1.8	0.2	1.1	0.0	0.9	0.7	1.4
66.....	1.5	0.7	1.3	0.0	0.5	0.7	1.4
67.....	1.7	0.5	1.3	0.0	1.7	0.3	0.3
68.....	1.5	0.7	1.7	0.0	1.3	0.8	1.7
69.....	1.5	0.5	1.2	0.0	0.6	1.1	1.2
70.....	1.3	0.5	1.2	— .2	1.0	1.2	1.5

TABLE 2—Continued.

Culture	Dextrose	Lactose	Saccharose	Mannite	Raffinose	Inulin	Salicin
71.....	1.5	0.6	1.0	0.0	0.7	0.8	1.3
72.....	1.4	1.6	1.1	0.0	1.0	0.1	0.8
73.....	1.4	0.5	1.0	0.0	0.5	0.7	1.0
74.....	1.9	0.5	1.2	0.0	0.6	0.1	1.3
75.....	1.3	0.5	0.9	0.0	0.3	0.0	1.2
76.....	1.5	0.5	0.9	0.0	0.5	1.3	0.5
77.....	1.4	1.7	1.4	— .3	0.6	0.2	1.2
78.....	3.8	1.9	2.4	1.6	0.5	0.2	2.0
79.....	1.5	0.5	1.5	0.0	1.2	0.2	0.7
80.....	1.4	1.5	1.2	0.0	0.9	1.4	0.9
81.....	3.8	2.0	2.6	1.5	0.5	0.3	1.8
82.....	1.2	0.5	0.9	0.0	1.1	1.0	0.5
83.....	1.5	0.1	1.1	0.0	1.1	1.2	0.9
84.....	1.5	0.3	1.5	0.7	1.0	0.2	1.2
85.....	2.1	0.7	2.0	0.4	1.3	1.7	1.4
86.....	1.6	0.5	1.5	0.1	0.5	1.4	1.8
87.....	1.8	0.4	1.0	0.9	0.7	2.5	1.4
88.....	1.6	0.6	1.4	0.9	0.5	2.6	1.3
89.....	1.9	0.6	0.8	0.2	0.6	0.6	2.3
90.....	1.6	0.3	1.6	— .2	0.7	1.4	0.9
91.....	1.6	2.6	2.6	1.0	0.3	3.8	1.6
92.....	1.6	0.5	1.1	0.0	0.7	0.6	1.1
93.....	1.8	0.5	1.0	0.1	0.4	1.0	0.6
94.....	1.7	0.3	1.5	0.5	0.5	0.8	1.0
95.....	1.9	1.7	1.4	— .1	1.1	1.1	1.2
96.....	1.7	0.5	0.7	— .1	0.8	0.5	0.5
97.....	1.5	0.3	0.9	0.1	0.3	1.7	0.5
98.....	1.8	0.3	1.7	0.0	0.4	1.7	1.7
99.....	1.9	1.7	1.3	1.2	0.6	0.8	0.8
100.....	1.1	1.7	0.5	0.2	0.9	1.1	1.0
101.....	1.7	1.3	1.6	1.0	0.9	2.3	1.4
102.....	— .3	0.3	1.5	— .1	0.2	0.7	0.9
103.....	1.4	1.7	0.5	0.0	1.1	0.8	0.7
104.....	2.3	1.9	1.6	1.3	1.3	1.5	2.0
105.....	3.1	0.7	2.3	1.2	2.6	3.2	— .3
106.....	1.4	0.3	1.4	— .2	0.4	0.5	1.3
107.....	0.6	0.4	0.6	0.8	0.4	0.6	0.5
108.....	1.5	0.5	1.6	0.0	0.5	2.1	0.9
109.....	1.5	0.2	1.4	0.0	0.0	0.5	1.7
110.....	0.9	1.1	1.0	— .1	0.6	0.7	1.0
111.....	1.5	2.3	1.8	— .2	0.7	1.3	0.9
112.....	1.7	0.6	1.7	0.0	0.5	0.5	1.4
113.....	1.0	0.6	0.5	0.5	0.1	0.5	0.6
114.....	1.2	0.5	0.8	0.0	0.4	1.6	0.5
115.....	1.6	0.4	1.7	0.1	0.5	1.9	1.0
116.....	1.7	0.7	1.7	— .4	1.2	0.6	1.7
117.....	1.5	0.6	0.9	— .1	0.4	0.5	1.2
118.....	1.6	0.5	1.2	— .2	0.4	1.1	1.1
119.....	1.4	0.5	1.3	— .1	0.7	0.9	0.3
120.....	0.8	0.4	0.9	0.7	0.7	0.4	0.8
121.....	1.0	0.6	1.2	0.2	0.2	0.3	0.7
122.....	1.2	0.5	0.6	0.1	0.2	0.6	0.5
123.....	1.4	0.9	1.3	— .3	0.3	0.7	0.5
124.....	1.7	0.5	1.6	— .1	0.4	1.4	0.8
125.....	1.4	0.4	1.1	0.1	0.8	0.6	1.0
126.....	0.8	0.7	0.7	— .1	0.5	0.7	0.5
127.....	0.2	0.7	0.4	0.1	0.1	0.8	0.8
128.....	0.9	0.6	0.5	0.4	0.4	0.5	0.0
129.....	1.6	0.9	1.6	0.4	0.8	1.3	0.0

throats and from milk. His results on the whole compare very favorably with those of Winslow and Broadhurst.

With the exception of the papers by Winslow and Palmer and by Houston in 1910 most of the research upon streptococci has been made primarily to determine the systematic relationships of these organisms rather than to formulate a practical method for the

differentiation of streptococci characteristic of human and animal excreta. In the majority of these investigations the cultures were isolated from milk, normal and diseased throats, and various streptococcic infections of tissues.

The studies upon streptococci presented in this paper were undertaken with the idea of following out Winslow's suggestion for using the fermentative action of streptococci in carbohydrate media as a means of differentiating between human and animal pollution in water supplies. In these experiments we have confined ourselves entirely to the study of fecal strains and have sought to approach the problem from the viewpoint of the sanitary water analyst rather than with the idea of adding to the knowledge concerning the systematic relationships of the group streptococci. In order to familiarize ourselves with Winslow's technic and at the same time to supply additional data for purposes of comparison we have tested the fermentative action of a number of cultures of fecal streptococci upon several carbohydrates before proceeding to the application of the test to the routine examination of water. This paper presents a preliminary report on the study of 350 strains of streptococci isolated from human, equine, and bovine feces. One hundred and twenty-three of these cultures were obtained from human feces; 129 from horse dung; and 98 from cow dung.

METHODS.

The methods used in these experiments are essentially those employed by Winslow and Broadhurst. The media used were prepared from beef extract by the usual method and the reaction adjusted to 0.5 per cent plus to the phenolphthalein scale. The cultures were isolated by plating direct on plain agar without previous enrichment in broth. After 24 hours' incubation at 37.5° C. characteristic colonies were transferred to tubes of maltose broth, and agar slope cultures made at the same time. If, after 24 hours' incubation, the agar slopes developed a scanty veil-like growth and the microscopic examination of the growths in the broth tubes revealed the presence of chains of spherical cells, the cultures were considered positive. Transfers were then made into broth containing one per cent of the carbohydrate used. The fermentative properties of the strains isolated from human feces were tested in dextrose, lactose, saccharose, raffinose, and mannite broth. Those isolated from the horse and cow were tested in broth containing the above carbohydrates and in inulin and salicin broth in addition. After three days' incubation at 37.5° C. the acidity produced through the decomposition of the various carbohydrates was determined by titration.¹ Five cubic centimeters of

¹ Our acknowledgments are due to Miss L. W. Fox and Mr. C. I. Nelson for assistance in some of the titrations for acidity.

TABLE 3.

SHOWING THE ACIDITY PRODUCED BY STREPTOCOCCI ISOLATED FROM COW DUNG IN DEXTROSE, LACTOSE, SACCAROSE, MANNITE, RAFFINOSE, INULIN, AND SALICIN BROTH.

Culture	Dextrose	Lactose	Saccharose	Mannite	Raffinose	Inulin	Salicin
1.....	2.0	2.2	1.6	1.4	1.8	2.7	1.7
2.....	0.6	0.6	0.2	0.6	0.4	0.6	0.6
3.....	1.8	1.9	0.8	2.3	1.7	2.7	2.4
4.....	1.8	2.0	1.9	0.3	1.9	3.1	1.6
5.....	1.9	3.1	0.8	0.4	1.6	2.9	2.0
6.....	2.0	2.5	2.0	0.1	1.7	2.8	1.3
7.....	2.3	2.2	1.7	0.2	1.9	1.7	1.6
8.....	2.3	2.1	0.8	0.5	1.7	1.5	1.7
9.....	1.9	1.9	1.6	0.2	1.7	2.2	1.5
10.....	1.6	2.0	0.8	0.1	1.2	2.3	1.7
11.....	1.7	0.8	0.3	0.3	0.3	0.7	0.6
12.....	2.5	1.0	1.8	0.6	1.5	2.2	1.7
13.....	0.6	1.6	1.9	0.1	1.6	1.7	2.2
14.....	0.7	0.6	0.3	0.7	— 1	0.4	0.4
15.....	2.0	1.8	1.7	— 1	1.8	2.0	2.1
16.....	0.6	0.6	0.0	0.1	0.3	0.8	0.5
17.....	2.0	1.9	1.6	0.0	1.4	1.7	2.2
18.....	2.0	2.0	2.0	0.0	1.4	1.6	1.9
19.....	3.1	2.2	2.2	0.1	0.5	0.7	1.4
20.....	0.5	0.6	0.2	0.1	0.2	0.7	0.5
21.....	1.6	1.9	1.7	0.2	1.5	2.7	2.3
22.....	4.4	4.0	4.3	0.5	3.3	4.0	4.5
23.....	2.1	1.0	1.5	0.2	2.0	2.7	1.8
24.....	1.9	1.8	1.7	0.1	— 4	2.8	1.7
25.....	2.5	2.4	1.9	0.3	2.0	3.1	2.3
26.....	1.9	1.9	1.8	0.5	1.3	3.5	1.7
27.....	2.3	2.5	1.7	0.2	1.4	3.3	2.1
28.....	2.9	3.0	1.6	0.1	1.7	2.8	1.7
29.....	2.8	2.3	1.5	0.3	1.4	2.0	1.5
30.....	2.2	2.2	1.8	0.5	1.5	2.8	1.5
31.....	0.9	0.8	0.5	0.4	1.0	0.2	0.7
32.....	2.9	2.1	2.7	0.2	3.0	2.6	3.0
33.....	2.2	2.1	2.5	0.2	2.3	3.4	2.6
34.....	4.3	3.0	2.6	0.3	3.3	0.4	2.0
35.....	2.6	2.2	2.6	0.1	2.6	3.4	2.6
36.....	2.1	2.1	2.6	0.3	3.1	2.7	2.5
37.....	1.9	2.2	2.5	0.3	3.2	3.7	2.4
38.....	2.2	2.5	3.0	0.5	3.3	2.5	2.9
39.....	2.0	2.0	2.6	0.4	4.3	3.7	2.7
40.....	1.6	1.8	2.5	1.3	0.6	0.9	1.5
41.....	2.9	2.4	2.8	0.2	3.2	3.8	2.8
42.....	2.8	3.0	2.8	0.2	3.7	2.8	3.0
43.....	1.7	0.7	1.3	1.0	0.9	1.0	1.1
44.....	2.8	1.6	2.8	0.6	3.2	3.7	2.8
45.....	1.3	1.2	1.1	0.9	1.1	1.1	1.3
46.....	1.1	0.8	1.1	1.3	1.2	1.3	1.2
47.....	3.2	2.1	3.6	1.5	0.4	1.7	2.0
48.....	0.4	0.4	0.1	0.2	0.1	0.3	0.2
49.....	2.6	2.4	2.3	0.8	2.4	2.9	2.9
50.....	2.4	2.8	2.4	1.0	3.0	3.1	2.6
51.....	0.7	0.7	0.4	0.6	0.4	0.2	0.2
52.....	3.0	2.6	2.4	0.6	2.8	2.7	3.2
53.....	2.4	2.0	2.3	0.7	2.6	3.5	2.0
54.....	0.4	0.4	0.1	0.1	0.0	0.7	0.3
55.....	2.8	2.4	2.3	0.5	2.6	3.4	2.7
56.....	2.2	2.5	2.4	2.5	2.3	2.6	2.4
57.....	2.2	1.5	2.3	0.5	2.3	3.5	2.5
58.....	2.7	2.5	2.6	0.6	2.5	2.9	2.3
59.....	2.8	2.5	3.0	0.7	2.7	3.6	0.9
60.....	2.5	2.5	2.3	0.7	1.9	2.6	2.4
61.....	2.9	2.4	2.6	0.8	3.4	3.1	2.2
62.....	2.4	2.1	2.5	0.8	3.5	3.2	2.8
63.....	2.5	2.6	2.0	0.6	2.6	2.6	2.6
64.....	2.1	2.7	2.6	0.7	2.1	3.1	3.0
65.....	2.9	2.2	2.7	0.7	2.3	3.0	2.0
66.....	2.4	2.4	2.6	0.7	2.0	2.7	2.2
67.....	2.4	2.4	2.7	1.0	2.7	3.8	3.0
68.....	2.0	1.7	1.9	0.2	2.0	2.3	1.6
69.....	1.5	1.8	2.1	0.4	1.9	2.7	1.6
70.....	1.4	1.5	2.1	0.2	2.5	1.6	2.0

TABLE 3—Continued.

Culture	Dextrose	Lactose	Saccharose	Mannite	Raffinose	Inulin	Salicin
71.....	2.3	2.2	2.5	0.5	2.9	3.5	1.7
72.....	1.0	1.8	2.4	0.5	2.9	3.0	1.7
73.....	1.8	2.0	2.1	0.0	2.4	3.0	1.9
74.....	0.8	1.8	2.3	0.3	2.8	3.2	1.7
75.....	1.5	1.8	1.8	0.2	1.9	3.0	1.7
76.....	3.2	1.5	0.6	1.6	0.3	1.5	1.1
77.....	1.7	1.6	2.2	0.0	2.5	2.9	1.8
78.....	1.0	1.2	1.5	0.6	0.4	0.5	0.6
79.....	1.6	1.7	2.2	0.0	2.5	3.0	1.8
80.....	1.6	0.9	2.2	0.0	2.2	2.7	0.8
81.....	0.5	1.4	1.0	0.7	0.7	0.5	0.6
82.....	1.7	1.6	1.8	0.0	2.6	3.2	1.8
83.....	1.5	1.7	2.3	0.1	2.3	3.3	1.6
84.....	1.5	1.7	2.3	0.1	2.4	3.4	1.6
85.....	1.8	1.8	1.5	0.3	2.8	2.9	1.3
86.....	1.9	1.0	2.1	0.3	2.7	3.8	1.8
87.....	1.7	1.7	1.7	0.1	2.0	3.1	0.7
88.....	0.5	1.3	2.5	0.2	2.2	2.7	0.6
89.....	0.4	0.2	0.2	0.1	1.1	0.4	0.2
90.....	1.8	1.2	2.0	0.1	1.9	2.7	1.7
91.....	1.7	1.3	1.9	0.4	1.9	3.2	0.3
92.....	1.9	1.1	2.4	0.5	2.0	2.9	1.7
93.....	0.4	0.1	0.0	0.0	0.2	0.5	0.2
94.....	2.9	0.0	1.0	0.9	0.3	0.3	0.3
95.....	1.9	2.1	1.1	0.5	1.0	2.5	1.7
96.....	2.5	1.7	3.0	0.2	2.4	2.4	1.0
97.....	0.9	0.8	1.1	0.7	0.5	1.0	1.7

the broth culture were measured by means of a graduated pipette and added to 45 c.c. of distilled water in a 250 c.c. Phillips beaker. The acidity was then determined by titrating in the cold against an N/20 sodium hydrate solution using phenolphthalein as an indicator. During the process of titration the flasks were placed on white porcelain slabs and the faintest indication of a pink color in the solution was taken as the end point. Uninoculated tubes of broth were incubated and titrated with each series of inoculations made in order to keep a check upon the reaction of media used. The milk and neutral red tests recommended by the English observers were not employed in this work as it seemed to the writers that the information gained by the study of the coagulation of milk and the reduction of neutral red does not furnish important additional data for the differentiation of the various types of streptococci studied in these experiments.

RESULTS.

The results of the titrations to determine the acidities produced by streptococci isolated from human, equine, and bovine excreta are given in detail in Tables 1, 2, and 3. These tables give the actual acidity produced by the cultures after deducting the initial acidity of the medium in which they were grown. From the study of these figures it will be observed that the amount of acid produced by streptococci isolated from the same species does not vary greatly in a given carbohydrate. For example 91 out of 123 strains isolated from human feces produced between three and four per

cent of acid in dextrose broth. One hundred and two out of 129 strains from horse dung produced between 0.5 per cent and 2.0 per cent of acid in saccharose broth. And 90 out of 98 strains from cow dung produced less than one per cent of acid in a mannite broth. These relationships are brought out more clearly in Table 4 and in Charts 1, 2, 3, 4, and 5, which have been plotted from it. In this table the range of acidity produced by the fecal streptococci, extending from -0.5 to 5.5 per cent normal acid, is expressed by a series of groups, each group representing one-half per cent of acid.

TABLE 4.
STREPTOCOCCI GROUPED ACCORDING TO THE PERCENTAGE OF ACID PRODUCED IN DIFFERENT CARBOHYDRATE MEDIA.

	- .5 0	0.1 0.5	0.6 1.0	1.1 1.5	1.6 2.0	2.1 2.5	2.6 3.0	3.1 3.5	3.6 4.0	4.1 4.5	4.6 5.0	5.1 5.5
	(Human 123)											
Dextrose.....	0	2	0	0	2	7	13	34	40	0	1	1
Lactose.....	3	2	0	7	38	42	9	1	0	0	0	0
Saccharose.....	8	0	2	16	53	19	2	0	0	0	0	1
Mannite.....	9	0	4	72	13	2	0	0	0	0	0	0
Raffinose.....	36	45	14	1	0	0	0	0	0	0	0	0
	(Horse 129)											
Dextrose.....	1	1	6	46	29	5	2	2	5	2	1	1
Lactose.....	1	46	23	8	13	3	4	1	0	0	0	0
Saccharose.....	0	5	24	39	16	5	5	4	0	0	0	0
Mannite.....	55	38	7	5	2	2	0	0	0	0	0	0
Raffinose.....	1	39	40	13	4	4	2	0	0	0	0	0
Inulin.....	6	29	22	21	8	5	3	3	2	0	0	0
Salicin.....	3	12	23	36	14	6	5	1	0	0	0	0
	(Cow 98)											
Dextrose.....	0	6	9	6	32	22	15	3	0	2	0	0
Lactose.....	2	4	12	10	31	29	7	1	1	0	0	0
Saccharose.....	2	9	6	9	25	27	17	0	1	1	0	0
Mannite.....	9	57	25	4	1	2	0	0	0	0	0	0
Raffinose.....	3	13	5	12	22	16	15	9	1	1	0	0
Inulin.....	0	11	9	4	8	7	33	20	7	0	0	0
Salicin.....	0	11	10	11	34	15	15	1	0	1	0	0

The percentage of strains from each species is placed in the acidity-group which corresponds to the amount of acid produced in each of the carbohydrates tested. By this arrangement it is possible to see at a glance where the center of fermentative activity lies and to determine the frequency with which certain definite amounts of acid are produced in a series of strains from a given source in a given carbohydrate. It is evident that the cultures arrange themselves into two quite distinct groups of numerical frequency. One group was found in the neighborhood of no acid or of slight acid production, and the second at a point from one to three per cent higher

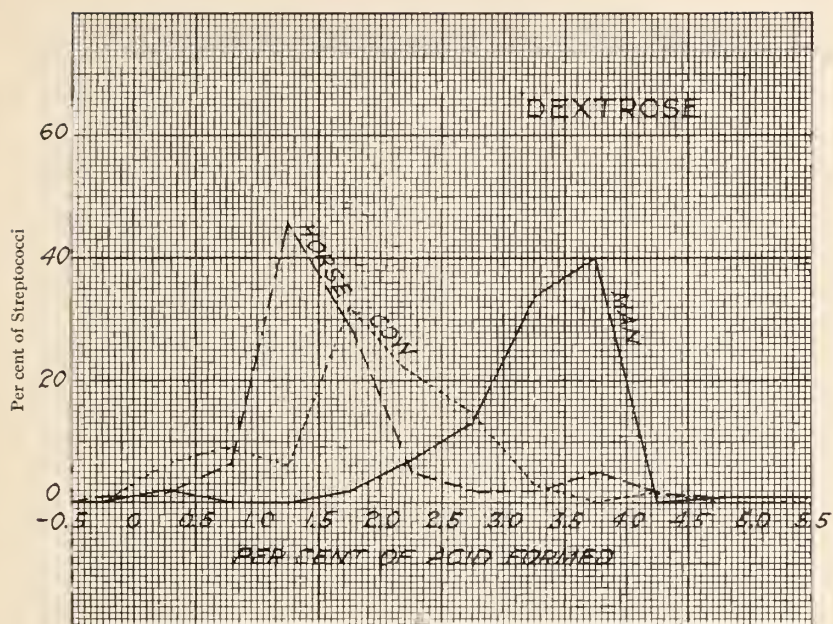


CHART 1.

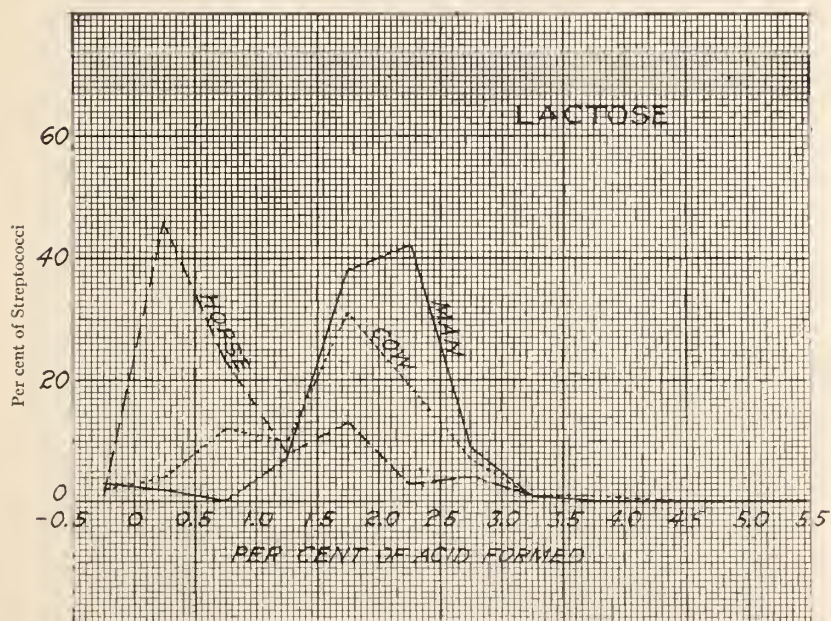


CHART 2.

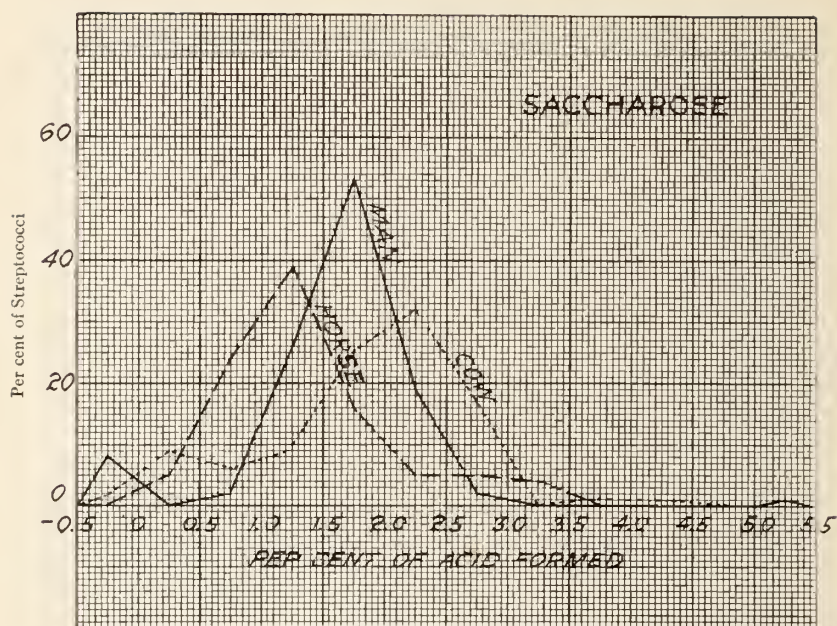


CHART 3

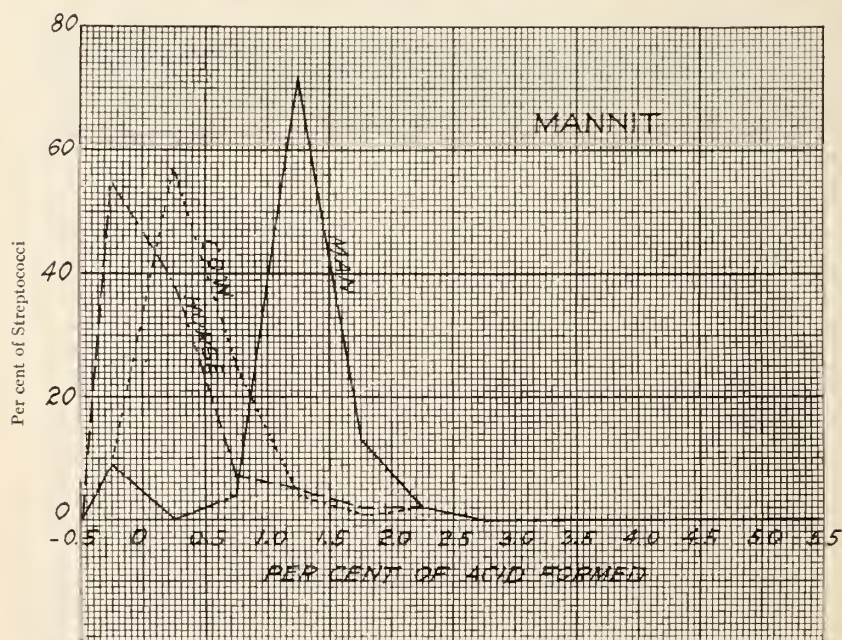


CHART 4

in the scale of acidity. (See accompanying charts.) The same general condition was noted in each of the carbohydrates studied by us. This point was brought out by Winslow, Broadhurst, and Hilliard, who have shown that these two groups represent probably a non-fermenting and a fermenting type of organism. The division line between these two types in all cases falls between 0.5 and 2.0 per cent normal; thus, following the suggestion of Winslow, we have

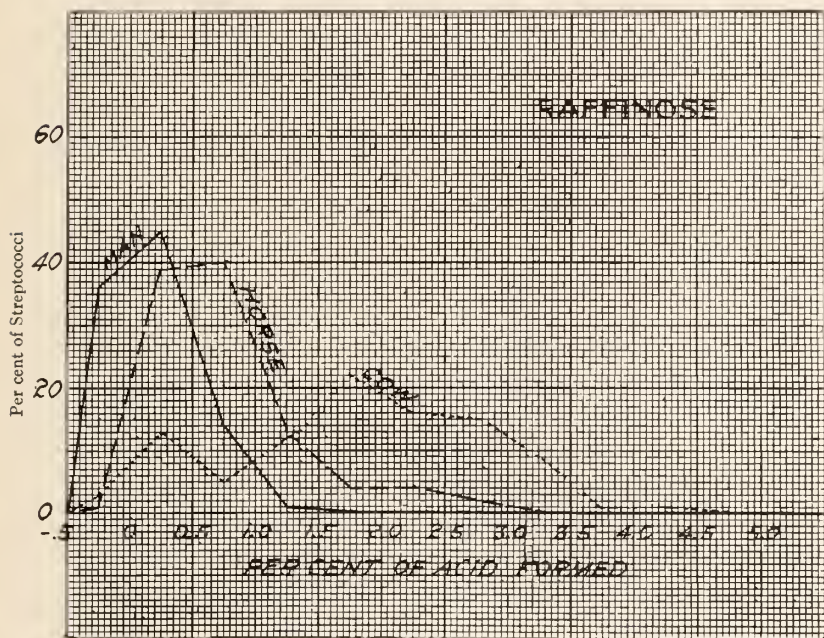


CHART 5.

considered as fermenting organisms only those which have produced over 1.2 per cent acidity. Those which produced 1.2 per cent or less we have classed among the non-fermenting types.

Our results show that dextrose is fermented by practically all strains of streptococci studied; 97 per cent of those isolated from human feces, 75 per cent of those from horse dung, and 81 per cent of those from cow dung acted upon this sugar. But the cultures from the human feces produced a considerably higher acidity than those from either the horse or cow. The great majority of human

strains produced between 3.5 per cent and 4 per cent of acid in this sugar; about 75 per cent of the streptococci from the horse produced between 1.0 per cent and 2.0 per cent; and 76 per cent of the cultures isolated from the cow produced between 1.5 per cent and 3.5 per cent of acid in dextrose.

Lactose is not attacked as readily as dextrose except possibly by streptococci from human and bovine feces. Ninety-four per cent of the human and 77 per cent of the bovine strains ferment lactose. There are comparatively few lactose fermenting streptococci in the excreta of the horse, only 24 per cent of the strains from this source being able to attack this sugar. The percentage of acid produced in lactose by streptococci from all these sources is not as high as in dextrose.

Saccharose is fermented by 90 per cent of the strains obtained from human feces, by 46 per cent of those from the horse, and by 75 per cent of those from the cow.

The streptococci that ferment mannite are found abundantly only in human feces. Sixty-five per cent of the cultures derived from this source fermented mannite more or less vigorously, while only 2 per cent of those from horse manure and 3 per cent of those from cow dung decomposed this substance. We have noted the presence of mannite fermenting streptococci in human feces in a consistently larger number of cases than is reported by Winslow.

No strains which fermented raffinose were isolated from human feces and only 12 per cent of those obtained from horse manure attacked this sugar. On the other hand, 73 per cent of the streptococci from cow dung fermented raffinose. In this respect also we obtained a larger number of raffinose fermenting streptococci from the cow than is noted by Winslow in his work. These results are not essentially different from those of either Winslow or Houston. Houston has omitted entirely the study of the action of streptococci on dextrose and Winslow has not tried out saccharose.

Table 5 shows the percentage of strains of streptococci fermenting different carbohydrates as found by Winslow, Houston, and Fuller and Armstrong.

Winslow has shown that the production of an acidity of 0.8 per cent in neutral litmus broth is sufficient to cause a distinct redding

of the litmus. Thus if the mere formation of acid is regarded as a positive test for fermentation the English observers have probably recorded as fermenting strains a number which would be considered non-fermenters if judged upon the basis of quantitative acid production. The somewhat higher figures recorded by Houston, Winslow attributes to the use of litmus as an indicator of the fermentative powers of the organisms tested.

TABLE 5.

CARBOHYDRATE	HUMAN			HORSE			COW		
	Winslow	Houston	Fuller and Armstrong	Winslow	Houston	Fuller and Armstrong	Winslow	Houston	Fuller and Armstrong
Dextrose.....	89	95	65	75	84	81
Lactose.....	62	100	94	8	0	24	52	100	77
Saccharose.....	85	90	46	94	75
Mannite.....	28	24	65	2	0	2	6	0	3
Raffinose.....	6	32	0	4	0	12	28	74	73

We find, as did Winslow, that dextrose is fermented by a larger number of the strains tested than any other sugar. A larger number of strains fermenting lactose was found by us than is reported by Winslow. In this respect our results show a closer agreement with those of Houston. We find also a higher percentage of streptococci in the cow dung capable of fermenting raffinose than is noted by Winslow. In this regard also our results are more nearly like those of the English observers. Houston reports 74 per cent of raffinose fermenting streptococci in cow dung, Winslow 28 per cent, and Fuller and Armstrong 75 per cent. The most important point of disagreement between our results and those of the other observers is in regard to the per cent of mannite fermenting organisms found in human feces. The per cent of mannite fermenting strains found by us in horse and cow dung corresponds closely to that noted by Winslow and Houston; but in the human feces we find them nearly three times as numerous as is found by either of the other observers. We recorded 65 per cent of mannite fermenting streptococci in human feces where Winslow and Houston find 28 per cent and 24 per cent respectively.

By correlating the results of the fermentation tests in each

separate carbohydrate several distinct types of streptococci can be quite clearly distinguished. We find for example that there is one group of organisms characterized by the fermentation of dextrose only, another characterized by the fermentation of dextrose and lactose, and a third by the fermentation of dextrose, lactose, and raffinose, etc. These results are expressed in the following table together with those published by Winslow in 1910.

TABLE 6.

SHOWING THE PERCENTAGE OF STRAINS FERMENTING DIFFERENT GROUPS OF SUGARS AS REPORTED BY WINSLOW, ANDREWES AND HORDER, AND FULLER AND ARMSTRONG.

CARBOHYDRATE FERMENTED	SPECIES	HUMAN		HORSE		COW	
	Andrewes and Horder's Types	Winslow	Fuller and Armstrong	Winslow	Fuller and Armstrong	Winslow	Fuller and Armstrong
None.....		9	3	15	13	18	14
Dextrose only.....	Equinus	23	3	73	55	27	4
Lactose only.....		2	0	0	1	5	1
Dextrose and Lactose.....	Mitis	31	30	5	18	21	5
Dextrose and Raffinose.....		0	0	3	7	3	4
Lactose and Raffinose.....		0	0	0	0	12	2
Dextrose, Lactose and Raffinose.....	Salivarius	5	0	0	4	9	64
Dextrose, Lactose and Mannite.....	Fecalis	23	65	0	1	2	2
All four.....		0	0	1	0	3	3

For purposes of comparison we will for the moment consider the fermentative action of these cultures in dextrose, lactose, mannite, and raffinose only, since these carbohydrates were used by Winslow in his work. The more prevalent types are those isolated from horse dung fermenting dextrose only; two strains isolated from human feces, one of which ferments dextrose and lactose and the other which ferments dextrose, lactose, and mannite; and a group isolated from cow dung which ferments dextrose, lactose, and raffinose. From examination of Table 6 it will be seen that the numerical frequency with which these types were found to occur varies somewhat from that reported by Winslow.

Winslow's work indicates a considerably wider distribution of streptococci fermenting dextrose only than is indicated by our results. This observer found that 23 per cent of the human strains, 73 per cent of equine, and 27 per cent of bovine strains fermented dextrose only. This type would correspond to Andrewes and

Horder's *Str. equinus*. Our results show the presence of only 3 per cent of this type in human feces, 4 per cent in cow dung, and 55 per cent in horse dung. We have isolated this dextrose fermenting *Str. equinus* in considerable numbers in excreta of the horse only.

Dextrose and lactose fermenters, corresponding to Andrewes and Horder's *Str. mitis*, were found to be quite numerous in human feces, less so in horse dung, and in very small numbers in cow dung. Thirty per cent of the human strains fell in this group, 18 per cent of the equine, and 5 per cent of the bovine strains. Winslow's results correspond very closely with ours in this regard, 31 per cent of his cultures belonging to this type. He found this type to be less numerous in horse dung and in considerably larger numbers in cow dung than is indicated by our results. Winslow reports only 5 per cent of equine strains belonging to this group as against 18 per cent recorded by us and 21 per cent of bovine strains as against 5 per cent.

Streptococci fermenting dextrose, lactose, and raffinose, corresponding to Andrewes and Horder's *Str. salivarius*, were found abundantly in the excreta of cows but not in the feces of any of the other species examined. Sixty-four of the strains isolated from cow dung are of this type. No streptococci corresponding to this type were found in human feces and but 4 per cent of those obtained from the horse belongs to this group. Winslow, on the other hand, finds that 5 per cent of the human strains fermented dextrose, lactose, and raffinose; no organisms of this type were found in horse dung and only 9 per cent of the bovine strains fermented these sugars. Our results indicate the presence of a much larger number of streptococci of this type in cow dung than has been observed by Winslow.

Strains which ferment dextrose, lactose, and mannite were abundant in human feces. Sixty-five of the streptococci isolated from this source are included in this class which corresponds to Andrewes and Horder's type *Str. fecalis*. One per cent of the strains from the horse dung and 2 per cent from cow dung are of this type. We are in close agreement with Winslow as to the distribution of this group of streptococci in the excreta of the horse and cow, but find that it is far more abundant in human feces than is reported by this observer. Sixty-five per cent of the strains from the human

feces fermented dextrose, lactose, and mannite while Winslow includes in this group but 23 per cent of the cultures isolated from man.

The four types of streptococci described above are relatively more abundant in feces than any others. Several other types, however, are observed with less frequency.

One group found in the excreta of all the species examined failed to ferment any of the carbohydrates tested. A like group of non-fermenting organisms was also noted by Winslow. Strains which fermented lactose alone are not abundant. None were observed in human feces. One per cent of the strains from the horse and one per cent of those from the cow fermented saccharose only. Winslow reports 2 per cent of the human and 5 per cent of bovine strains which attack lactose only. None of these types were isolated from horse dung.

Organisms fermenting dextrose and raffinose were found in small numbers in the feces of both the horse and the cow by Winslow and ourselves.

Organisms fermenting lactose and raffinose were observed in cow dung but not in cultures from human and equine feces.

A few streptococci fermenting dextrose, lactose, mannite, and raffinose were found in cow dung but not in human and equine excreta. One per cent of the cultures isolated by Winslow from horse dung fermented dextrose, lactose, mannite, and raffinose. If we take into consideration the action of these organisms upon saccharose, very little change is found to be made in the main type centers recorded in the above correlations. By the introduction of saccharose into these calculations 15 groups appear. The type fermenting dextrose only (*Str. equinus*) is broken up into two groups, one fermenting dextrose only and the other fermenting dextrose and saccharose, the latter corresponding to the *Str. equinus* of Andrewes and Horder. The other important groups remain practically the same. The results obtained from the fermentation of inulin and salicin by fecal streptococci have not been included in this work for the reason that these tests were not introduced until after the experiments were well under way and therefore we have no records of the action of human strains upon these sugars.

SUMMARY.

From the results of these experiments it appears:

1. That streptococci producing a high acidity in dextrose media are in general characteristic of human excreta. These strains which produce between 3.5 per cent and 4.0 per cent acidity in this medium are relatively abundant in human excreta. Dextrose fermenting strains are less abundant in the excreta of the horse and cow, and produce an acidity considerably less than that of the human strains.

2. That streptococci fermenting mannite are present in human feces in large numbers but are almost entirely lacking in the excreta of the horse and the cow. Strains fermenting dextrose, lactose, and mannite (Andrewes and Horder's *Str. fecalis*) comprise 65 per cent of the cultures isolated from human stools, while only 1 per cent of the strains from the horse and 2 per cent of those from the cow are of this type.

3. That streptococci fermenting lactose are comparatively rare in horse dung.

4. That streptococci fermenting raffinose are abundant only in cow dung. Sixty-four per cent of the strains isolated from this source ferment dextrose, lactose, and raffinose (Andrewes and Horder's *Str. salivarius*), 2 per cent ferment lactose and raffinose, and 4 per cent, dextrose and raffinose. Streptococci of the salivarius type were not observed in human excreta, and comprised but 4 per cent of the strains isolated from horse dung.

CONCLUSIONS.

The bacterial flora of human feces is characterized in general by the presence of streptococci producing between 3.5 per cent and 4.0 per cent of acid in dextrose media. The prevailing type is *Str. fecalis* which ferments dextrose, lactose, and mannite. *Str. mitis* which ferments dextrose and lactose is also found in considerable numbers.

Horse dung contains few streptococci which ferment lactose. The predominating type is *Str. equinus* which ferments dextrose and saccharose.

The streptococci of cow dung are characterized by their power to ferment raffinose. The majority of strains isolated from this source are of the *Str. salivarius* type which ferments dextrose, lactose, and raffinose.

These results accord in general with those of Winslow and Palmer. They differ chiefly in the numerical frequency with which certain types occur in the different species examined. For example, Winslow reports that but 23 per cent of the strains isolated from human feces are of the *Str. fecalis* type, while our results indicate that a much larger proportion (65 per cent) of the strains from this material are of this type fermenting dextrose, lactose, and mannite. Winslow reports that 73 per cent of the equine streptococci belong to the *Str. equinus* type while but 55 per cent of the strains isolated by us from horse dung are of this type. Winslow reports but 9 per cent of *Str. salivarius* in cow dung while our tests indicate the presence of this type in 64 per cent of the strains isolated from this source.

Further experiments are now in progress with the view to testing the practical value of the fermentative activities of fecal streptococci in carbohydrate media for the differentiation of human and animal pollution in water supplies. In this work we are using the water samples received in the State Hygienic Laboratory for routine analysis.

SPIROCHAETA SUI, ITS SIGNIFICANCE AS A PATHOGENIC ORGANISM.*

STUDIES ON HOG CHOLERA.

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(WITH PLATE 4.)

INTRODUCTION.

Since results of the dark field study of the blood of animals suffering from hog cholera were reported,¹ the work has been continued and several new phases of the problem have been investigated. In the former article it was reported that a relatively large spirochete and characteristic granules were present in the blood of 40 hogs infected with cholera, representing 12 different strains of virus, while the blood of 48 normal hogs was observed to be free from spirochetes and granules. The spirochetes observed in specimens of blood from cholera hogs were very few in number, but the characteristic granules were apparently always present during the fastigium and were regarded as important findings.

In conjunction with the later studies of this spirochete in the blood, many observations were made by means of the dark field of material taken from the intestinal ulcers of hogs dead from hog cholera. Dark field examinations have been made of the necrotic tissue from the intestinal ulcers of all animals dead from hog cholera during the course of our experiments of the last six months. Scrapings from the intestinal mucosa and contents of the crypts in the ceca of normal hogs, some of these being susceptible to hog cholera, others immune to the disease, were also examined at every opportunity. In all, the study with the dark field of material from the crypts and ulcers of normal and diseased hogs has included about 140 cases. As a result of this work, the details of which will be published separately, we are inclined to believe that the following observations are true.

* Received for publication August 11, 1913.

¹ King, Baeslack, and Hoffmann, *Jour. Infect. Dis.*, 1913, 12, p. 206.

1. The mucosa of the large intestine, particularly that of the cecum, of normal hogs, includes in its flora non-pathogenic spirilla and spirochetes, relatively large forms predominating.

2. In the ulcerated patches of cecal mucosa and in the crypts, near the ileo-cecal valve, of hogs dead from cholera is localized a constant species of spirochete, which is pathogenic for swine and which plays an important part in the production of hog cholera.

3. The crypts and healed ulcers of hogs actively immunized against hog cholera may contain, for a variable period of time after immunization, in addition to non-pathogenic spirochetes, the same species of pathogenic spirochete referred to above.

There are many factors entering into the above tentative conclusions which cannot be discussed in detail in this paper, but which will follow in future reports of our studies on this disease. Among the more important observations, however, are the results obtained from the experimental curative treatment of cases of hog cholera with arsenical and mercurial preparations. This work resulted, not in cures, but, usually, in prolongation of life, which, in most cases, was coincident with an apparent decrease in number or total disappearance of the spirochetes in question from the necrotic tissue in the cecal ulcers. Positive dark field findings of spirochetes in intestinal ulcers of cholera hogs are in accordance with the results reported by von R  ther,¹ Uhlenhuth and Haendel,² and Betegh.³ The presence of spirochetes in the bile of cholera hogs, as reported by Uhlenhuth and Haendel, has not been found in the cases examined thus far.

On account of the fact that efforts to secure cultures or satisfactory stained preparations of *Spirochaeta suis* from the blood of cholera hogs were not successful, and because of the large amount of bacterial contamination present in the intestinal lesions, attempts were made to find localized foci of the organism elsewhere in the diseased body. Microscopical preparations and cultures were made from the spleen, liver, lymphatic glands, spinal cord, cerebrospinal fluid, bile, and urine. These attempts were unsuccessful except in one instance. In one case (Hog 615, inoculated subcu-

¹ *Berl. tierarztl. Wchnschr.*, 1911, 27, p. 191.

² *Handbuch d. path. Mikroorg.*, 1913, 6, p. 337.

³ *Berl. tierarztl. Wchnschr.*, 1912, 28, p. 968.

taneously with exudate from local lesion, Hog 599), the dark field revealed in the inguinal lymph gland a spirochete resembling the assumed pathogenic form. In many of the dark field preparations numerous granules were observed, but it was impossible to draw any definite conclusions as to their origin and relationship to *Spirochaeta suis* or to tissue elements.

Finally, when an opportunity presented itself, dark field examinations were made of external lesions on the leg and ear of a case of cholera of the chronic type (Hog 583, May 8, 1913) and they were found to contain spirochetes, presumably of one species, in large numbers. So far as we know, similar observations have never been reported except by Dodd¹ of Pretoria, Transvaal. In describing "a disease of the pig due to a spirochete," Dodd states that he found a spirochete in the cutaneous lesions of a number of pigs. The disease could not be transmitted experimentally by the inoculation of blood from an infected animal, but was transmitted, however, by actual contact. The infection was fatal to several pigs. Autopsy revealed nothing except the local skin lesions and general anemia. The disease appeared to be some cutaneous infection due to the spirochete found in the local lesions. Scrapings from the skin of healthy hogs and from the normal skin of the affected pigs were found to contain no spirochetes.

Since the first observation we have not only found that all external local lesions examined, including 11 cases of hog cholera, were constant foci for *Spirochaeta suis*, but we have also been able to produce typical hog cholera, with local external lesions, by inoculating a small bit of the tissue or a small amount of exudate from local lesions of the affected animal into a healthy pig. Scrapings from the healthy skin of hogs and from the areas of discoloration on the ventral surface of the body of animals suffering from cholera have been submitted to dark field examination with negative results. The results of this work, which is still in progress, will be published later.

In our former paper, the spirochete observed in the blood of hogs infected with hog cholera was described as follows:

"In the specimens of blood from all infected hogs, which have been observed by means of the dark field, a relatively large spirochete has been found. It averages

¹ *Jour. Comp. Path. and Ther.*, 1906, 19, p. 216.

from five to seven microns in length and one micron in width. The body of the organism is flexible and round at its ends. It presents no knobbed appearance at its poles. Actively motile, it revolves about its longitudinal axis. Its motility is undulating in character and its spirals are fixed. A few of these organisms have been observed dividing longitudinally. In one permanent microscopical mount, prepared by india ink fixation, one of these organisms apparently shows a polar flagellum. On the dark field this spirochete is readily distinguished from bacteria on account of its lack of rigidity and its characteristic motility, and from 'blood filaments' by its greater refractive properties and characteristic morphology.

"This spirochete has not been found in large numbers, in any of the blood preparations. However, in nearly every specimen examined, more than one has been observed and in many cases five or six have been found with little difficulty. As a rule the organisms have been found to be more numerous at the height of the disease. The specimens of blood examined have been diluted in the proportion of about one to ten or fifteen with sterile sodium citrate solution, which factor should be considered in contemplating the number observed in a given positive specimen. Moreover, it is suggested that this organism, when observed as a spirochete form, constitutes only one stage of its development."

This description appears to be correct, except that we have never verified the presence of a polar flagellum.

It must be conceded that any successful dark field investigation requires patient work and much practice in interpretation. Therefore, one unacquainted with the dark field should not expect to obtain immediate satisfactory results from the study of blood. An intimate course of study is necessary to enable one to differentiate between "filaments," artefacts, and organisms of the morphological nature of the spirochete described in the blood of cholera hogs.¹

The last statement quoted we now accept as a conclusion rather than a suggestion. Results lead us to believe that the spirochete observed in few numbers in the blood of cholera hogs is a transitional form.

The spirochete, which is constantly observed in the ulcerated mucosa and crypts in the ceca and in the external local lesions of cholera-infected animals, is an actively motile, comparatively long, spiral-shaped organism, of slight thickness. Measurements show that it is approximately 6-10 microns in length, averaging about

¹ In this connection we were especially fortunate in the collaboration of our colleague, Dr. F. W. Baeslack. Because of his experience with the dark field in his study of *Spirochaeta pallidum* (see *Jour. Infect. Dis.*, 1913, 12, p. 55) and in his experimental observations of certain Trypanosomes, our dark field study of hog cholera blood was placed to great advantage. It is a pleasure to acknowledge his valuable assistance in that work, as well as the many helpful suggestions he has offered in our present investigation.

7 or 8 microns, and 0.3-0.6 micron in width. The convolutions vary from three to ten in number, rarely more than eight, and the spirals are fixed. It has been observed to divide longitudinally, and morphological variations, such as described by Gleitsmann¹ as a part of the life cycle of the Sudanspirochaetae, have been observed.

In our former report on the dark field study of the blood, observations relative to the presence of granules were summarized as follows:

"Certain types of granules appear to be characteristic of blood from cholera hogs. It usually contains many granules, some very fine yet more distinct than blood dust, some larger still, and some very distinct highly refractive bodies. In many specimens of cholera blood were observed innumerable small granules, which were much more definite and distinct in outline than blood dust, and easily differentiated from the whitish, partially refractive granules from ruptured leukocytes and from the more highly refractive and larger bodies composed of debris, bacteria, and filaments. In this work the presence of these granules appeared to be so characteristic of blood from cholera hogs that it became an invaluable aid in finding the spirochetes. While these particular bodies may be disintegrated blood elements resulting from disease processes, yet it may properly be suggested that some of them may represent certain stages in the life cycle of the spirochete which has been observed."

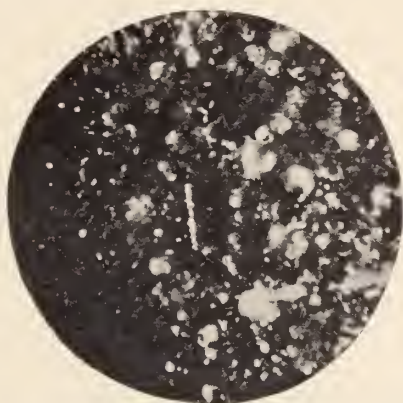


FIG. 1.—Photomicrograph of *Spirochaeta suis* in exudate, ear lesion, Hog 626. $\times 1200$. (Dark field preparation.)

As the result of continued investigations, we believe that the granules represent an important factor in the life history of *Spirochaeta suis*. References to similar observations in the study of various spirochetes are found in the writings of Balfour,² Mühlens,³ Ross,⁴ McDonagh,⁵ Dutton,⁶ Mayer,⁷ Mackinnon,⁸ Schellack,⁹ Bosanquet,¹⁰ Marchoux and Couvy,¹¹ and others.

¹ *Centralbl. f. Bakteriöl.*, I, Orig., 1913, 68, p. 31.

² *Report IV, Wellcome Research Lab.*, p. 76.

³ *Handbuch d. Path. Protozoen*, Prowazek, Leipzig, 1912, p. 361.

⁴ *Brit. Med. Jour.*, 1912, p. 1651.

⁶ *Jour. Trop. Med.*, 1907, 10, p. 385.

⁵ *Lancet, Proc. Roy. Soc.*, 1913, 6, p. 86.

⁷ *Arch. f. Schiffsz.-u. Tropenhyg.*, 1908, 12, Beihefte 1, p. 1.

⁸ *Parasitology*, 1909, 2, p. 267.

⁹ *Arch. a. d. k. Gsndhtsamte*, 1909, 30, p. 351.

¹⁰ *Spirochaetes*, Saunders, 1911.

¹¹ *Ann. de l'Inst. Past.*, 1913, 27, p. 450.

In cultures made from material containing *Spirochaeta suis* the presence of small granular forms is quite constant. As these appear in dark field preparations from cultures, they resemble those which were observed in the blood, and are seen in the exudate from local lesions, of cholera-infected hogs. They are relatively highly refractive, smaller than cocci, peculiarly distinct in appearance from foreign material, and possess more than the characteristic Brownian movement. The assumption that these granules form an integral part of the biologic processes of the organism, *Spirochaeta suis*, is substantiated by the results of the detailed experiments which follow.

Recognizing the difficulties involved in attempting to study *Spirochaeta suis*, and in determining its possible significance as a pathogenic form, our efforts have been directed toward the cultivation of the organism. This work has included the inoculation of artificial culture media with blood, intestinal ulcers, urine, contents of local external lesions, and other tissues from cases of hog cholera, followed by careful dark field examinations.

CULTIVATION OF SPIROCHAETA SUIS.¹

A great many plantings on a variety of artificial culture media have been made from the blood, intestinal ulcers, and external local lesions of cholera-infected animals. These cultures have been made chiefly in deep horse serum media, to which have been added various chemicals in different proportions. All have been grown under strict anaerobic conditions in special electric incubators. Various means were used in attempting to eliminate contaminating organisms, which were always present in cultures made from the ulcers and, to a lesser extent, in those made from material in local lesions.

The culture medium which proved most successful consisted of a mixture of horse serum and pyrogallic acid, in which was placed

¹ As the proof of this paper is being read we note an article in current literature by Pfeiler and Lentz (*Berl. tierärztl. Wchnschr.*, 1913, 29, p. 690), who present the case histories of 15 pigs which died after having received injections of cultures made from the Berkefeld filtrates of serum and tissue exudates from hog cholera infected animals. These cultures represented transfers in the first, second, third, and fourth generations. The authors carefully avoid any statements which might indicate the nature of the cultures. No mention is made of the character of the organism in their cultures, the composition of their culture medium, or how long or under what conditions the cultures were incubated. On account of the conspicuous lack of such important data one can form no conclusion as to their mode of procedure, and, hence, the results which they have published must be regarded with some skepticism.

fresh rabbit kidney at the time of inoculation. The pyrogallic medium of Proca, Danila, and Stroe¹ was made up in lots of one liter each by adding to 900 c.c. of horse serum 100 c.c. of pyrogallic acid mixture. The pyrogallic solution should be somewhat aged. A stock solution was therefore kept, which consisted of pyrogallic acid, 10 gm.; sodium hydroxid, 20 gm., and water, 1,000 c.c. After the addition of this mixture to horse serum and distribution in tubes the medium was heated one hour at 60° C. on each of three consecutive days.

While this pyrogallic serum medium does not eliminate all bacterial contamination, at the same time allowing the spirochete to grow, it was found to give better results than media containing formol, gentian violet, phenol, or other inhibitory substances. For uncontaminated material, such as Berkefeld filtrates of impure cultures of spirochetes, plain or ascitic horse serum media were utilized. Fresh rabbit kidney was added to all media used and care was exercised that the horse serum was only partially coagulated and soft in consistency when ready for use.

The cultures have been examined at different intervals of time, following inoculation and incubation, in order to detect any facts which might come to light concerning the life history, morphology, and physiological habits of the organism. The method of examining cultures consists simply in withdrawing, with a sterile capillary pipette, a small portion of culture from the lower layers of the medium and examining the same in microscopical mount by means of the dark field. The Giemsa method of staining is employed whenever permanent mounts are desired. After some practice one can readily detect, by the dark field, spirochetes and granules in fresh cultures in distinction from cocci, other bacterial contamination, crystals, and small particles of culture medium.

NOTES ON TRANSMISSION OF HOG CHOLERA BY MEANS OF CULTURES OF SPIROCHAETA SUI.

I. ORIGINAL CULTURE.

Culture 551-A was secured from the ulcerated cecum of Hog 551. The account of this case may be found in the chart for Hog 551.²

¹ *Compt. rend. Soc. de biol.*, 1912, 72, p. 895.

² Positive and negative signs indicate the presence or absence of *Spirochaeta suis* in the blood of the animal on dates indicated.

Cultures were made from the ulcerated cecum, spleen, and lymphatic glands of Hog 551. Those from the spleen and lymphatic glands developed no growth, while some of those from the cecum, after five days' incubation under anaerobic conditions, showed the growth of spirochetes which varied in form and size. Fig. 3, p. 212, in our former publication² shows a photomicrograph of a stained preparation made from this culture. When this culture was studied, our attention was directed especially to the larger forms of spirochetes which were found in it. This was due to the fact that we were familiar with the form which had been observed in the blood of cases of hog cholera. At this time no special attention was given to smaller forms representing more typical spirochetes which were present in this culture, as was shown, some weeks after the study of the culture, by careful re-examinations of the permanent stained preparations.

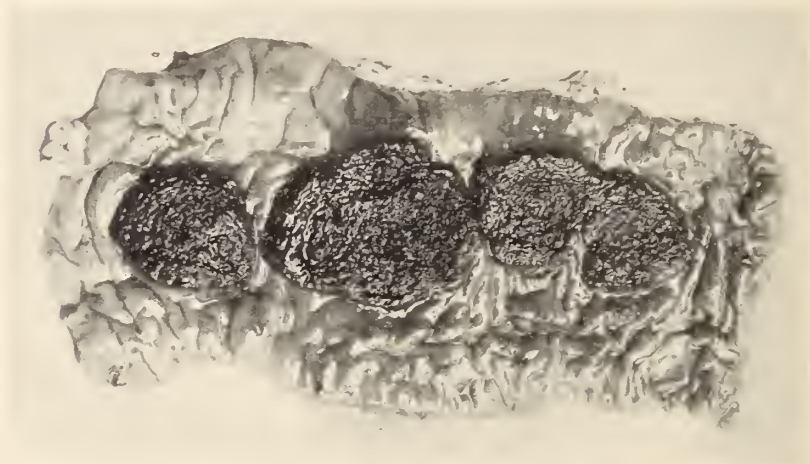


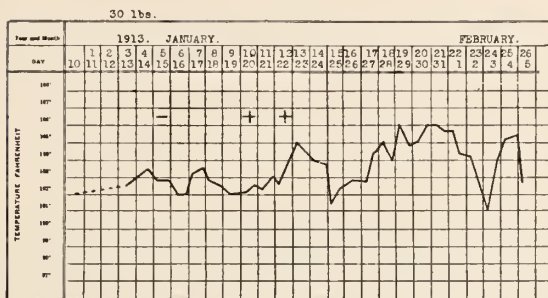
FIG. 2.—Ulcers in cecum of Hog 569.

Transfers were made from Culture 551-A and grown both aerobically and anaerobically. The species of bacteria which contaminated Culture 551-A were very carefully isolated and identified as follows: *B. pyocyaneus*, three strains; *B. coli communis*, five strains. These cultures, which contaminated 551-A, representing *B. coli communis* and *B. pyocyaneus*, were mixed and suspended in physiologic salt solution. Six cubic centimeters of this suspension on March 14 were injected intravenously into Hog 570. This animal showed no disposition to develop hog cholera, but did react to the injection of live *B. pyocyaneus* and *B. coli communis*, as the clinical chart for Hog 570 will show.

Culture 551-A was suspended in sterile physiologic salt solution and an intravenous injection of 10 c.c. given to Hog 559. The results of this inoculation are recorded in the chart for Hog 559.

² *Jour. Infect. Dis.*, 1913, 12, p. 206.

Hog 551.



January 10. Received from Rochester, Mich.

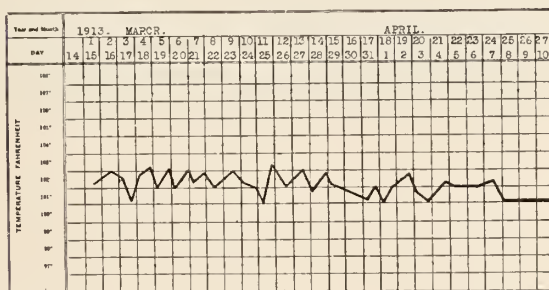
January 16. Intramuscular injection 8 c.c. of virus, Berkefeld filtered, 1:4 saline. Ohio (Pettigrew) strain.

January 31. Animal very weak.

February 5. Animal moribund, bled and examined.

Typical lesions of cholera: lungs, large areas of hepatization; kidney, petechiated, capsule non-adherent; lymphatic and inguinal glands, greatly enlarged and hemorrhagic; spleen, friable and engorged with blood, normal in size; cecum, large area of ulcers.

Hog 570.



March 14. Intravenous injection of 6 c.c. physiologic salt suspension cultures from cecum of Hog 559.

March 18-25. Slightly indisposed, appetite not good, otherwise condition practically normal.

March 31. Apparently in good condition. Released.

April 2. A little "off feed."

April 9. Fully recovered, released and put on other work April 14.

April 30. Animal found dead, result of intramuscular injection of 5 c.c. II Cycle virus. Typical lesions of hog cholera.

Hog 559 was in a perfectly healthy condition at the time of inoculation, February 10, and weighed approximately 75 pounds. Four days after inoculation, the temperature of the animal began to rise and on the fifth day symptoms of hog cholera appeared. The animal was slightly "off feed" and inclined to be inactive. Dark field examination of the blood at this time showed the presence of a few spirochetes and characteristic granules. After 48 hours the temperature dropped somewhat and for several days ranged between 103° and 104° . About 12 days after the inoculation the animal appeared to be brighter and seemed to be rapidly improving, but 15 days after inoculation a remission occurred, the temperature was elevated 2° during one afternoon, and



FIG. 3.—Hog 559.

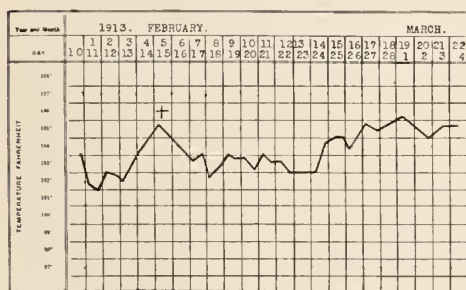
from this time the animal gradually grew worse until death, which occurred 22 days after inoculation. The later symptoms consisted of weakness, inability to use hind quarters, bloody urine, mucous diarrhea, anorexia, and general malaise. On March 4 the animal was moribund and, after being photographed, was bled from the carotid artery and examined (see photograph of Hog 559). On autopsy typical lesions of hog cholera were found.

Blood extravasations were present in the subcutaneous tissue, especially in the axillary region and lower abdominal region. Both the auricles and ventricles of the heart showed ecchymosis. The lungs contained numerous hemorrhagic infarcts and a few areas of hepatization. The liver appeared to be somewhat enlarged and contained areas of fatty degeneration. The spleen was slightly atrophied, tissue firm, but

engorged with blood. Both kidneys were full of petechiae. The mucosa of the cecum and the large intestine was congested and marked with hemorrhagic spots throughout. Two typical button ulcers were found in the cecum. In the small intestine there were present two enlarged areas, consisting of necrotic intestinal wall surrounded by extensive hemorrhages. The peritoneum in the lower abdominal region showed hemorrhagic areas. The mesenteric, inguinal, and intraperitoneal lymph glands were enlarged and hemorrhagic (see Plate 4).

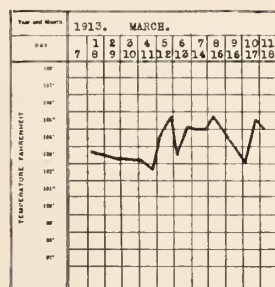
A specimen of blood serum from Hog 559 was filtered through Berkefeld, after being diluted 1:4 with physiologic salt solution, and 10 c.c. of the filtrate injected intramuscularly into Hog 569. This material proved to be virulent, as Hog 569 showed severe symptoms of hog cholera five days after inoculation and became moribund on the eleventh day. The autopsy showed classical lesions of hog cholera (see chart for Hog 569).

Hog 559.



- February 10. Intravenous injection 10 c.c. suspension physiological salt solution of Culture 551-A.
 February 15. Clinical symptoms present, appetite poor.
 February 23. Animal improved in condition.
 February 25. Remission.
 March 4. Animal moribund, and bled from carotid artery. Typical lesions: heart, lungs, kidney, cecum, and lymphatic glands.

Hog 569.



- March 7. Blood negative, free from granules.
 March 7. Intramuscular injection of 10 c.c. Virus 559, diluted 1:4, physiological salt and Berkefeld-filtered strain (Culture 551-A).
 March 12. Symptoms appeared.
 March 18. Animal moribund and bled from carotid artery. Lesions typical: heart, lungs, spleen, kidney, inguinal glands, and mesenteric lymph glands; ulcers in cecum.

With reference to infection from other material than that which was used in the inoculations, it should be stated that results from Hog 559, as well as all others used in this series of experiments, were carefully controlled by the presence of normal hogs in the experimental stable. The animals used in each series of experiments, as well as the normal animals, were kept in separate rooms. The normal animals were placed under exactly the same conditions and had the same care that was exercised in regard to the treated animals.

An additional control on this work depended upon the use of Hog 570, which was inoculated with the contamination present in Culture 551-A. Furthermore, it has been observed that the serum of Hog 559, filtered through Berkefeld, was capable of producing typical hog cholera in Hog 569.

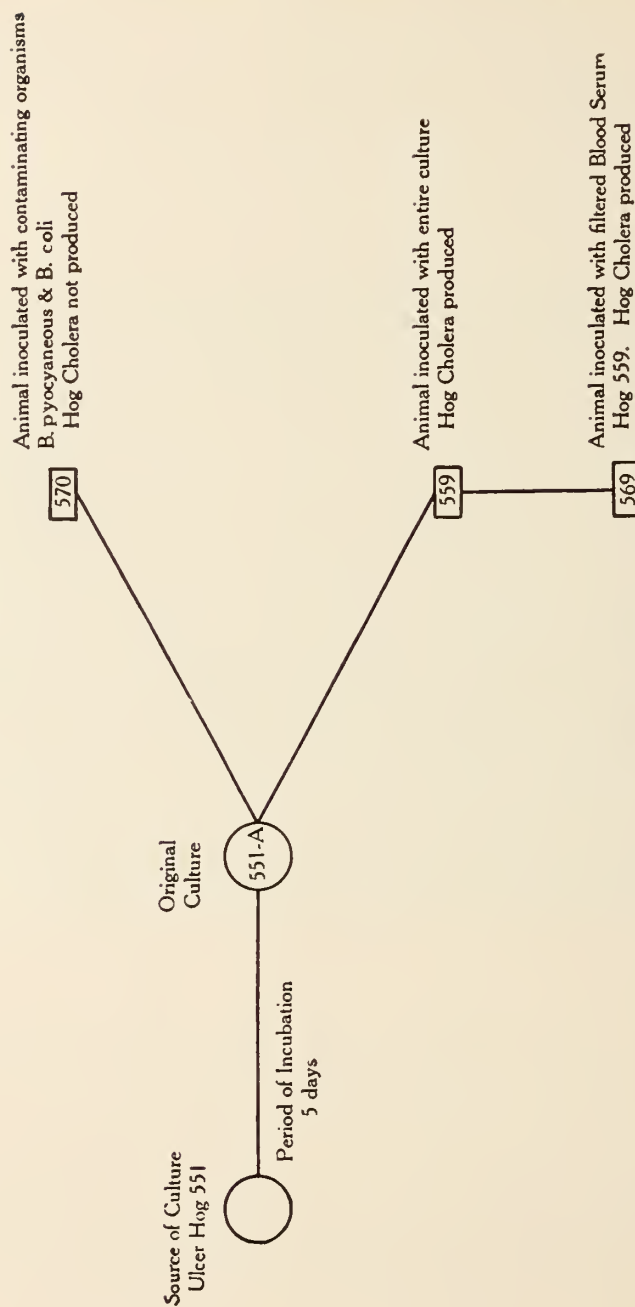


CHART 1.—Graphic representation of experiments with original culture from Hog 551.

Among the criticisms which may be offered on this experiment, the most important relates to the possibility of transferring the original virus from Hog 551 through Culture 551-A. This point could not be controlled, altho an estimate was made that Hog 559 received less than 1/750 c.c. of material originally obtained from the ulcer of Hog 551. It has been estimated by King and Wilson¹ that the minimum fatal dose of hog cholera virus, diluted in physiologic salt solution, is approximately 1/300 c.c. The above approximation that Hog 559 received about 1/750 c.c. was obtained on the basis of the utilization of about 1/15 gm. of ulcer from Hog 551 in approximately 10 c.c. of culture media. However, these estimates, including both the suggested minimum fatal dose of hog cholera virus in physiologic salt solution and the amount of original material transferred through Culture 551-A to Hog 559, are open to serious question in attempts to estimate whether or not any of the original virus from Hog 551 was transferred to Hog 559.

II. TRANSFER I. SECOND GENERATION ON ARTIFICIAL MEDIA.

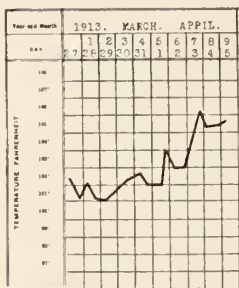
This culture was secured from the strain of virus from Hog 577. This animal received an intramuscular injection of 8 c.c. horse serum virus, diluted 1:4 in physiologic salt solution and Berkefeld filtered. The animal developed typical symptoms of hog cholera and after nine days, being moribund, was bled from the carotid artery and examined.

Hog 587 on April 11 received an intramuscular injection of 5 c.c. of serum from Hog 577. This animal promptly developed hog cholera of the acute type after five days' incubation and was bled and examined eight days after inoculation. The autopsy showed typical lesions of hog cholera of the acute type. Numerous blood extravasations were present in the subcutaneous tissue, especially in the axillary region. Both lungs were almost completely consolidated. The kidneys showed areas of cloudy swelling and petechiae near the upper ends. The spleen was engorged with blood. The mesenteric and inguinal glands were enlarged and hemorrhagic. The cecum contained a few small ulcers around the valve (see chart for Hog 587).

On April 19 cultures were made from the material obtained from the small ulcers in the cecum of Hog 587. These were incubated under anaerobic conditions for a period of 17 days and on May 5 dark field examination showed the presence of *Spirochaeta suis*. A variety of contaminating organisms was also present, both cocci and bacilli being represented in the contamination. The culture medium in the tube labeled "587-A" showed no liquefaction from the growth of the contaminating bacteria, and from it a number of transfers were made on May 5. On May 27, after incubation for 22 days, transfer labeled "Transfer 1-587-A," when submitted to dark field

¹ Bull. 171, Kansas State Agric. Coll., p. 163.

Hog 577.



March 27. Intramuscular injection 8 c.c. activated horse serum, diluted 1:4, physiological salt solution and Berkefeld filtered.

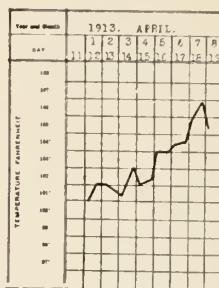
April 1. Symptoms appeared.

April 3. Animal very weak in hind quarters.

April 5. Animal moribund, bled and examined.

Typical lesions: heart, lungs, kidney, lymph glands, cecum; small intestine infected with *Ascaris suilla*.

Hog 587.



April 11. Intramuscular injection of 5 c.c. of Virus 577 I Cycle activated horse serum.

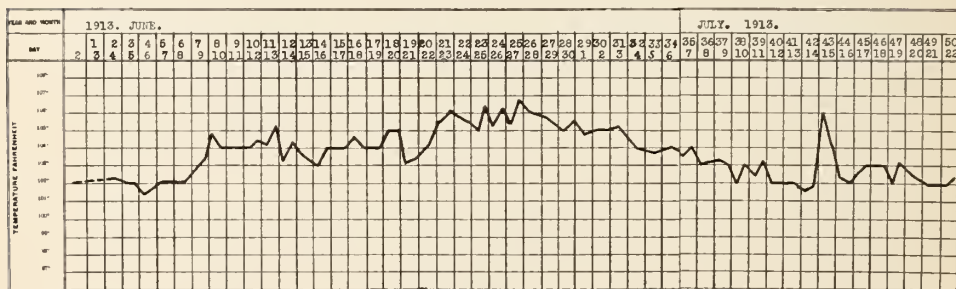
April 16. Symptoms of cholera appeared.

April 19. Animal moribund, bled from carotid artery and examined.

Lesions: lungs, spleen, cecum, subcutaneous tissue, and inguinal glands.

examination, showed a few *Spirochaetae suis* and numerous granules. All of the contaminating bacilli which were present in the original culture, 587-A, had disappeared and the only contamination present in the transfer was a micrococcus. A portion of this culture, Transfer 1-A-587-A, was broken up in sterile water and 1.5 c.c. of the suspension of this culture were inoculated, intramuscularly, into each of three hogs, Nos. 612, 613, and 614. Hogs 612 and 614, after eight days' incubation, developed symptoms of hog cholera. After a few days the disease assumed the chronic form.

Hog 612.



June 2. Intramuscular injection 1.5 c.c. suspension Culture Transfer 1-587.

June 10. Symptoms appeared.

June 10-July 1. Chronic case of cholera.

July 4. Condition improved.

July 15. Animal had been fighting with strange hogs.

July 22. Released as healthy immune.

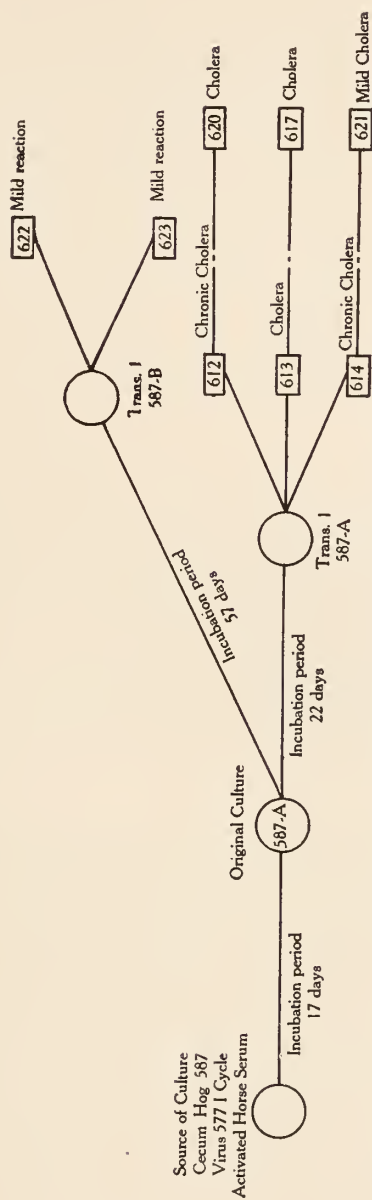
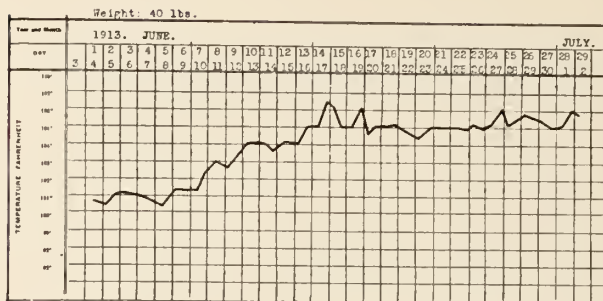


CHART 2.—Graphic representation of experiments with Transfer I-587.

HOG 613.



June 3. Intramuscular injection of 1.5 c.c. suspension Culture Transfer 1-587.

June 10. Symptoms appeared.

June 16-July 2. Subacute type of cholera.

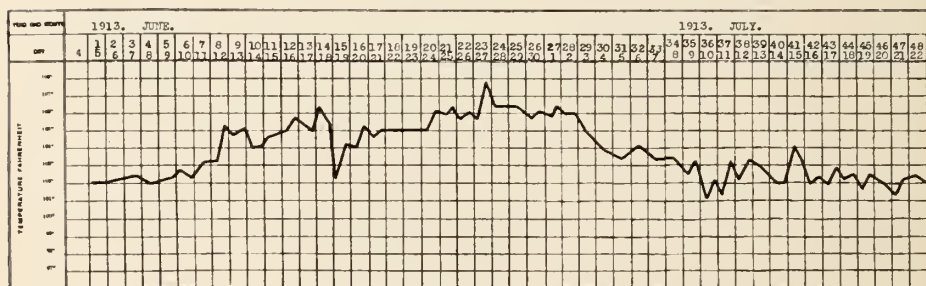
July 2. Animal moribund, killed and examined.

Typical lesions.

The animals lost their normal appetite and, during a period of about three weeks following the initial symptoms, became somewhat emaciated. On the fifth week after the inoculation of the animals, rapid convalescence took place and they fully regained normal condition. Both animals had been confined with cholera hogs, but following the experiment they remained healthy and were released as active immunes.

Hog 613 developed a more acute form of the disease than Hogs 612 and 614. After an incubation period of eight days, the temperature rapidly rose and maintained an elevation of approximately 106° - 107° for a period of two weeks. During this time Hog 613 manifested the usual symptoms of hog cholera, consisting of anorexia, general weakness and malaise, paralysis of hind quarters, emaciation, and constipation. On July 2, 29 days after inoculation, the animal was killed; on examination, characteristic

HOG 614.



June 4. Intramuscular injection 1.5 c.c. suspension Culture Transfer 1-578.

June 12. Animal "off feed."

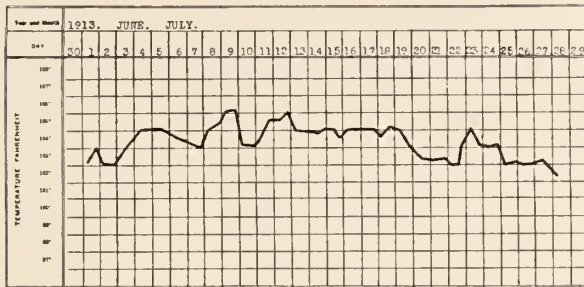
June 15-30. Symptoms of chronic type of cholera.

July 4. General improvement noted.

July 15. Animal had been fighting, placed with strange hogs.

July 22. Released as healthy immune.

Hog 620.

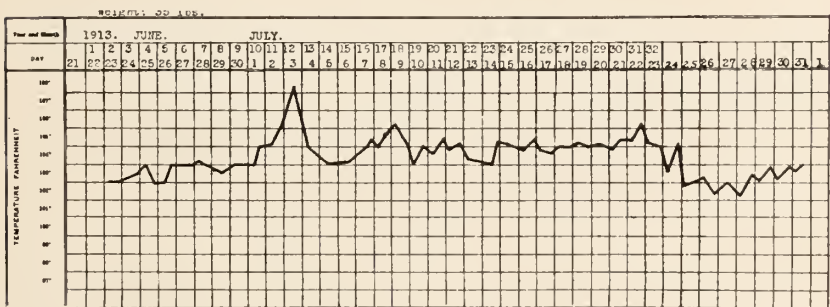


- June 30. Intramuscular injection of 10 c.c. of blood of Hog 612, diluted 1:4, and Berkefeld filtered.
 July 4-5. High temperature—may have been due to excessively hot weather.
 July 8. Symptoms of cholera.
 July 10-20. Gradual emaciation, constipation, anorexia, and malaise.
 July 21. Gradual return of appetite and slow convalescence.
 July 29. Apparently recovered.
 July 30. Animal released as normal.

lesions of hog cholera were found. The heart was normal, the lungs were congested and contained areas of hepatization. The spleen was enlarged, soft, and friable, and engorged with blood. The kidneys were petechiated. The mesenteric and lymphatic glands were enlarged and hemorrhagic, and the congested mucosa of the cecum contained small ulcerated patches near the ileocecal valve. *Spirochaeta suis* was present in the ulcers.

This experiment was controlled in the manner explained in the description of the inoculation of Hog 559 with original culture. Normal hogs were kept in the stable under the same conditions as were these animals. Hogs 612 and 614, which survived

Hog 617.



- June 21. Intramuscular injection of 12 c.c. of blood of Hog 613, diluted 1:4, and Berkefeld filtered.
 June 27. Symptoms appeared..
 July 1-15. Animal very weak, emaciated, constipated, anorexia, and malaise.
 July 20. Some improvement noted.

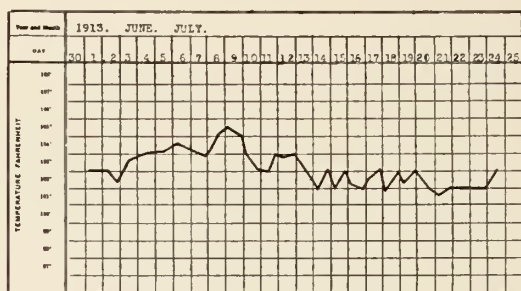
the experiment, possessed active immunity, as was demonstrated by the fact that they were placed with hogs sick with cholera two weeks before being released.

During the period of infection, a sample of blood was obtained from the tail of each of Hogs 612, 613, and 614, used in the experiment above in testing the pathogenicity of Transfer 1-587-A. These samples were defibrinated, diluted 1:4 in sterile water, and passed through the Berkefeld filter. Ten cubic centimeters of the diluted, Berkefeld-filtered blood from Hogs 612, 613, and 614 were injected intramuscularly into Hogs 620, 617, and 621, respectively.

Hog 620, which received the Berkefeld-filtered blood serum from Hog 612, developed symptoms of cholera eight days after inoculation. This case assumed the chronic type extending over two weeks' time after which the animal gradually became convalescent.

Hog 617, which received the Berkefeld-filtered blood from Hog 613 (the animal which developed the most acute form of the disease of the three cases described

Hog 621.



June 30. Intramuscular injection of 10 c.c. of blood of Hog 614, diluted 1:4, and Berkefeld filtered.

July 6-9. Animal not normal, appetite poor, general malaise.

July 10. Condition improved.

July 14. Condition normal.

July 25. Released as healthy immune.

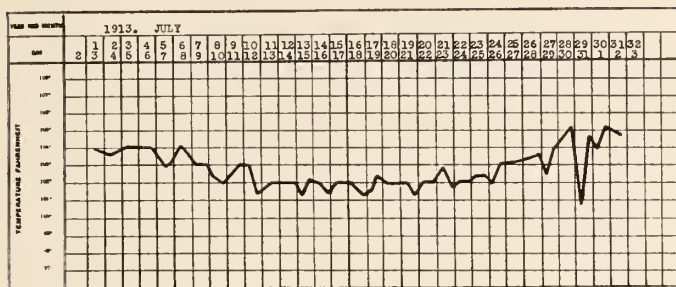
above), showed unmistakable signs of hog cholera about six days after inoculation. The course of the disease in this animal was of the chronic type and death occurred two months after inoculation.

Hog 621, inoculated with the Berkefeld-filtered serum of Hog 614, showed mild symptoms of cholera eight days after inoculation. After being slightly "off feed" for two or three days the animal regained its normal condition.

After the conclusion of this experiment, Hogs 620 and 621 were confined for several weeks in an infected inclosure with cholera pigs. No evidences of disease developed, thus establishing the fact that the animals possessed active immunity.

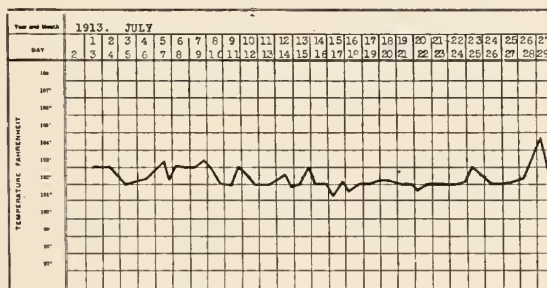
On July 1 one of the transfers, which was made from Strain 587 and which had been kept in the incubator since the time of inoculation, May 5, was submitted to dark field examination. It was found that the culture contained very few organisms which could be recognized as spirochetes, but many granules were present and the small amount of contamination consisted of a micrococcus. This culture, which had been incubated for a period of 57 days, was designated "Transfer 1-587-B," and two hogs,

HOG 622.



- July 2. Intramuscular injection of 10 c.c. unfiltered suspension of positive culture No. 587 (May 5, 1913)—(July 2, 1913).
- July 7-8. Appetite poor, general malaise, feces normal.
- July 9. Apparently normal.
- July 22. Exposed to hog cholera; placed with sick animals and injected intramuscularly with 2 c.c. of virus from Hog 631.
- July 29-August 2. Symptoms of paralysis and chorea. Appetite fairly good. Animal not emaciated.
- August 3. Animal found dead and examined.
- Typical lesions of hog cholera absent; intestinal mucosa normal. Culture made from edematous fluid of muscular tissue shows *Str. pyogenes* present.

HOG 623.



- July 2. Intramuscular injection of 10 c.c. unfiltered suspension of positive culture No. 587 (May 5, 1913)—(July 2, 1913).
- July 7-8. Appetite poor, general malaise, feces normal.
- July 9. Apparently normal.
- July 22. Exposed to hog cholera; placed with sick animals and injected intramuscularly with 2 c.c. of virus from Hog 631.
- July 29. Animal showed symptoms of paralysis and chorea. Could not walk.
- July 30. Found dead and examined.
- Mucous membrane of small and large intestine congested. No ulcers present in cecum.
- Typical lesions of cholera absent.

Nos. 622 and 623, were each injected intramuscularly with 10 c.c. of the suspension of this culture in sterile water. Six days after the inoculation of these animals, they showed, in addition to slight temperature changes, mild symptoms of illness. The appetite was poor and the animals were inclined to be listless and inactive. After about two days of inactivity and loss of appetite, they again resumed a normal condition and continued so until exposed to cholera.

On July 22 these animals were exposed to the disease by inoculation, natural exposure, and access to carcass of hog dead from cholera. On July 28 both hogs, Nos. 622 and 623, developed symptoms of paralysis and chorea. On July 29 Hog 623 was found dead and autopsy did not show lesions of hog cholera. Dark field examination of the mucosa of the cecum failed to show any spirochetes. Hog 622, on July 30, showed a moderate rise in temperature, but did not lose appetite or manifest other symptoms of cholera. The right front leg became enormously swollen. On August 3 Hog 622 was found dead. No characteristic lesions of hog cholera were present and cultures made from the serous fluid in the swollen leg developed *Str. pyogenes*. Several preparations were made from the mucosa of the cecum and were submitted to dark field examination with negative results.

On account of the absence of typical symptoms and lesions of hog cholera, also the course of the disease in the control animal (see notes below on Hog 641) and the presence of *Str. pyogenes* in the edematous tissue of Hog 622, it seemed probable that Hogs 622 and 623 died from infection other than hog cholera.

III. TRANSFER 2. THIRD GENERATION ON ARTIFICIAL MEDIA.

On May 27 a number of transfers were made from Culture Transfer 1-587-A. These cultures were incubated under anaerobic conditions from May 27 to July 2, when they were removed from the incubator and examined by the dark field method. One of these transfers, designated "Transfer 2-587-A," contained very little contamination represented by one species of micrococcus and, while only a few spirochetes could be found scattered through the medium, the culture contained many granules. This culture, Transfer 2-587-A, was macerated in sterile water, and on July 3, Hogs 627 and 628 were each inoculated, intramuscularly, with 10 c.c. of the suspension of this transfer. Five days after inoculation both of these animals showed indications of indisposition. From July 8 to July 12, the appetite was poor and the animals were inactive. On July 14 the condition of both hogs was much improved and on July 16 both were pronounced apparently healthy.

On July 22 these animals were exposed to hog cholera in the same manner as Hogs 622 and 623, described above. After seven days' incubation both animals developed symptoms of cholera. Hog 627 succumbed to the disease 22 days after exposure. Hog 628, after suffering from the disease in chronic form, gradually became convalescent and on September 1 was released. Both animals showed greater resistance and maintained a better clinical condition than did the control Hog 641.

On July 11, during the period in which Hog 628 was showing a general reaction from the injection of Transfer 2-587-A, a few cubic centimeters of blood were drawn from the tail, defibrinated, diluted 1:4 in sterile water, and filtered through a bacteria-proof Berkefeld. On July 15 Hog 635 received intramuscularly 5 c.c. of the Berkefeld-filtered serum, diluted 1:4, of Hog 628. This animal showed mild symptoms five days after inoculation and soon developed hog cholera of the subacute type. Four weeks after inoculation Hog 635 was found dead. Autopsy revealed typical lesions.

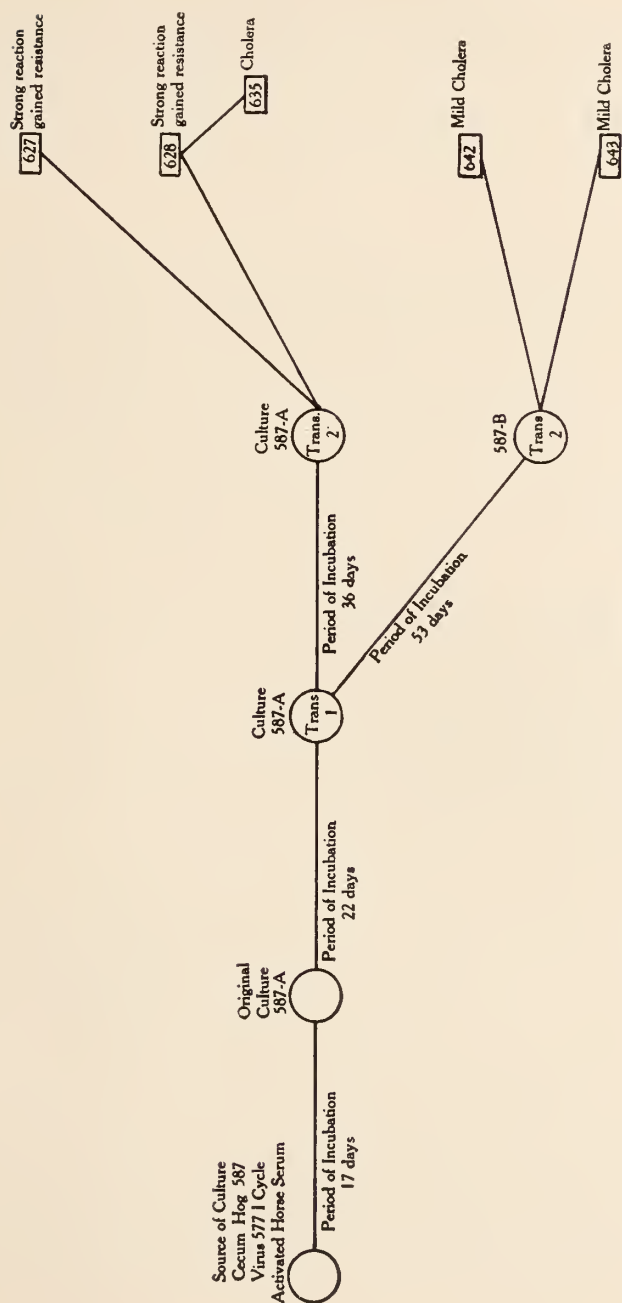
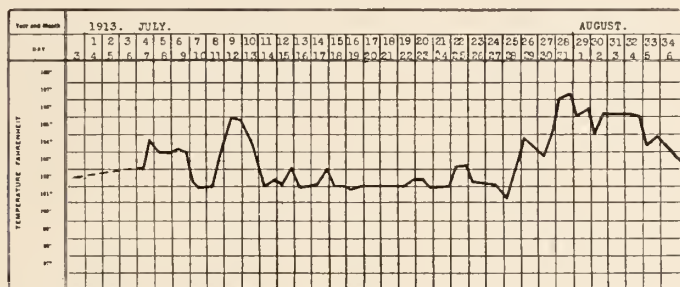


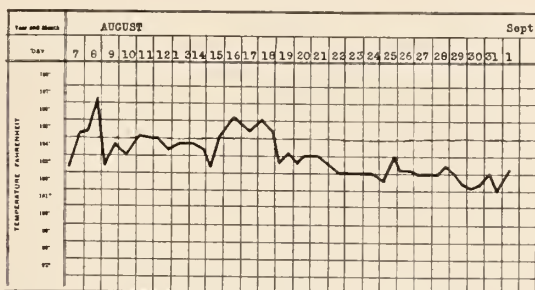
CHART 3.—Graphic representation of experiments with Transfer 2-587.

These hogs, Nos. 624 and 625, were exposed with control Hog 641 (see p. 482), by inoculation and by natural exposure. Three days after exposure Hog 624 showed evidences of illness, apparently due to gastritis and autointoxication, resulting from the ingestion of pus from an infected carcass. After a few days this animal fully recovered and exhibited a thrifty condition throughout the experiment, thus proving the existence of active immunity. Hog 625 showed scarcely any reaction as the

Hog 628.



Hog 628 (continued).

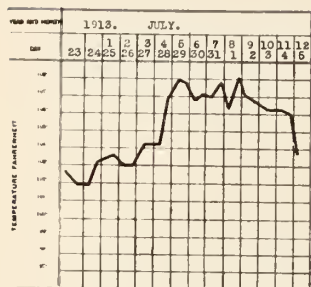


- July 3. Intramuscular injection of 10 c.c. unfiltered suspension culture No. 587 (May 27, 1913)
 —(July 3, 1913).
 July 8-13. Inactive, anorexia.
 July 15. Animal apparently normal.
 July 22. Intramuscular injection 2 c.c. Virus Strain 631.
 July 30-August 5. Symptoms of chronic type of cholera.
 August 6. Emaciated and growing progressively weaker.
 September 1. Animal normal, recovered and released.

result of the exposure, and was considered as being actively immunized by the filtered Culture Transfer 1-587-B.

On July 9, during the reaction following inoculation with filtered culture, a specimen of blood taken from the tail of Hog 625 was defibrinated, diluted 1:4 in sterile water, and filtered through the Berkefeld filter. On July 15, Hog 634 received intramuscularly 5 c.c. of the diluted, filtered blood from Hog 625. Five days after inoculation of Hog 634 the temperature rose two degrees, the animal was "off feed" and

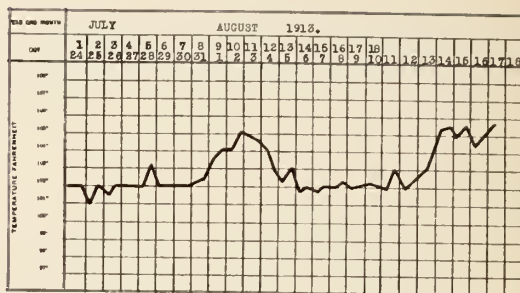
Hog 641.



- July 24. Intramuscular injection of 2 c.c. virus.
 July 29. Typical symptoms of hog cholera.
 August 5. Animal died.

Lesions: lungs, areas of hepatization; spleen, enlarged, soft and friable, engorged with blood; kidneys, petechiated; lymphatic and inguinal glands, enlarged and hemorrhagic; cecum, mucosa congested, small ulcers near ileocecal valve. *Spirochaeta suis* present.

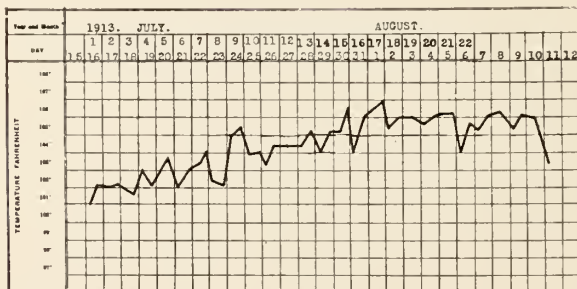
Hog 642.



- July 24. Intramuscular injection 5 c.c. physiologic salt suspension Culture 587 (May 27, 1913)—(July 20, 1913), Berkefeld filtered.
 August 1. Symptoms of anorexia; malaise, slight blepharitis.
 August 5. Animal normal.
 August 10. Temperature normal, tho the appearance was not normal.
 August 14. Indication of relapse; typical symptoms of cholera.
 August 18. Animal found dead.

Typical lesions of hog cholera; subcutaneous tissue, heart, lungs, spleen, kidney, lymphatic system, cecum; *Spirochaeta suis* present.

Hog 635.



- July 15. Intramuscular injection of 5 c.c. blood serum of Hog 628, diluted 1:4, and Berkefeld filtered.
 July 20-22. Animal "off feed," dull, inactive.
 July 24. Condition improved; appetite poor.
 July 29-August 5. Symptoms of subacute type of cholera.
 August 6. Nearly moribund.
 August 11. Animal died.

Typical lesions of hog cholera: lungs, heart, spleen, kidney, cecum, and lymph glands.

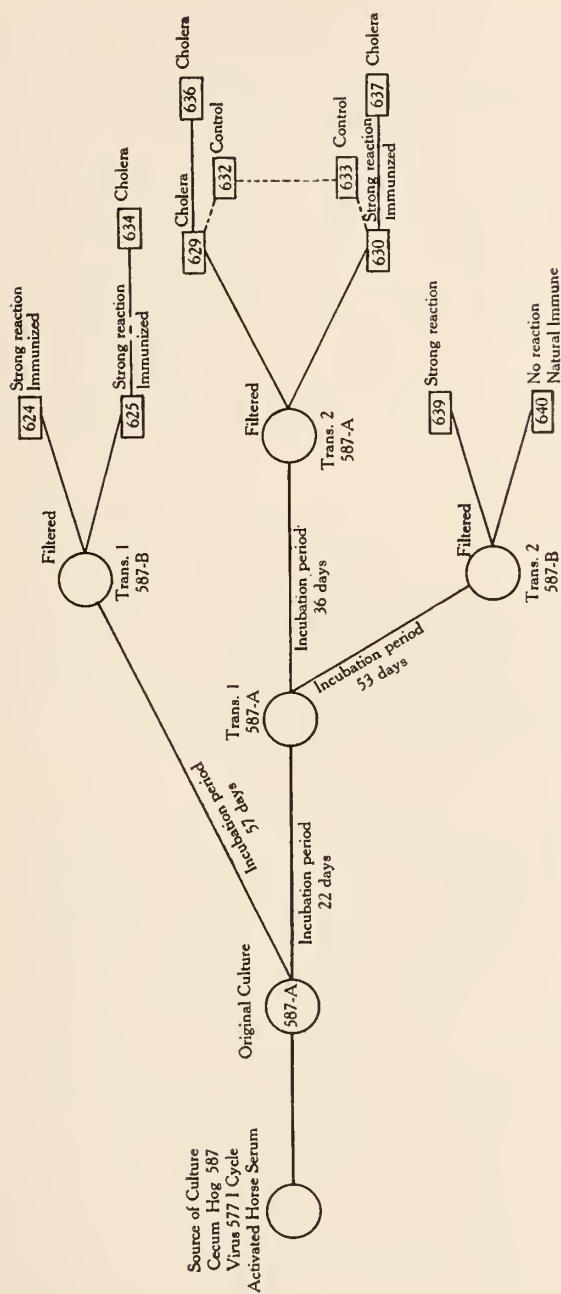
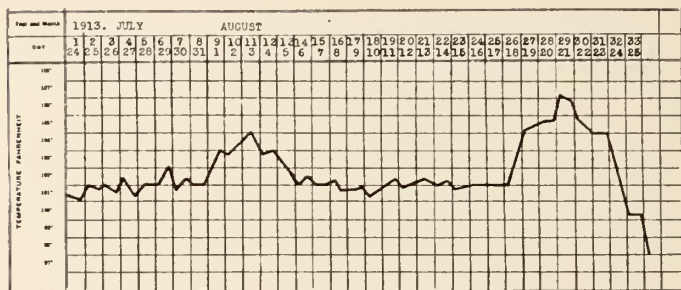


CHART 4.—Graphic representation of experiments with filtered cultures.

Hog 643.



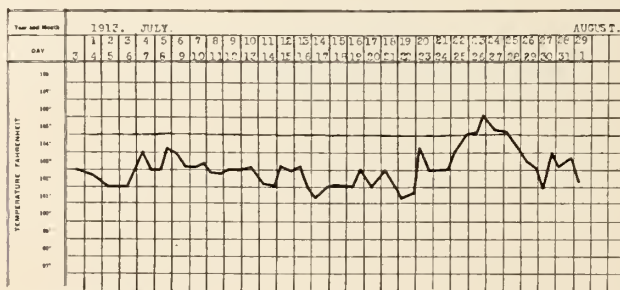
- July 24. Intramuscular injection 5 c.c. physiologic salt suspension Culture 587 (May 27, 1913)—(July 20, 1913), Berkefeld filtered.
- August 1. Symptoms of anorexia, malaise, slight blepharitis.
- August 5. Animal normal.
- August 10-17. Animal apparently normal.
- August 23. Typical case of hog cholera.
- August 26. Animal found dead.

Typical lesions of hog cholera: heart, lungs, spleen, kidney, lymphatic glands, subcutaneous tissue, and cecum.

became constipated. This animal developed cholera of the subacute type and was found dead about four weeks after inoculation.

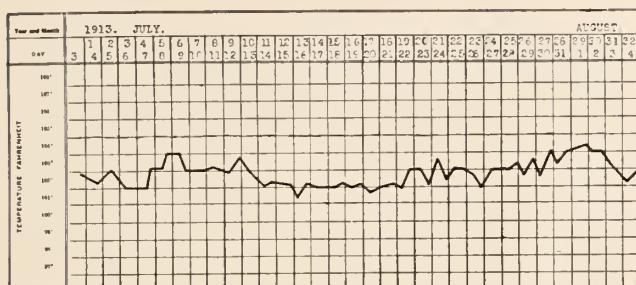
b) *Transfer 2.*—Culture Transfer 2-587-A, described above, on July 2 was filtered and treated in exactly the same manner as described concerning the Berkefeld filtration of Culture Transfer 1-587-B. Transfer 2-587-A had been grown in the incubator for 36 days. On July 3 Hogs 629 and 630 were each injected intramuscularly with

Hog 624.



- July 3. Intramuscular injection of 10 c.c. Culture 587 (May 5, 1913)—(July 2, 1913), diluted 1:10, and Berkefeld filtered.
- July 8-9. Anorexia, dull, listless.
- July 10. Condition improved.
- July 13. Animal apparently normal.
- July 22. Intramuscular injection 2 c.c. Virus Strain 631.
- July 23. Animal had been fighting with strange hogs.
- August 1. Animal normal. Released.

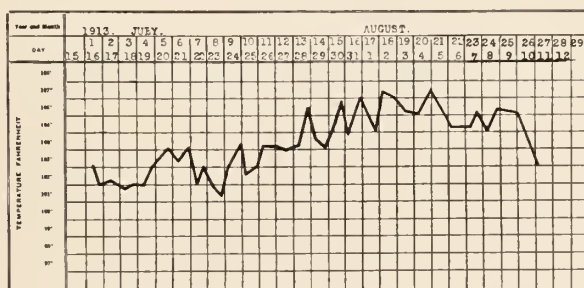
Hog 625.



- July 3. Intramuscular injection of 10 c.c. Culture 587 (May 5, 1913)–(July 2, 1913), diluted 1:10, and Berkefeld filtered.
 July 8–9. Poor appetite, eyes dull, inactive.
 July 14. Animal apparently normal.
 July 22. Intramuscular injection 2 c.c. Virus Strain 631.
 August 4. Animal released.

10 c.c. of Culture Transfer 2-587-A, diluted 1:10, and filtered through bacteria-proof Berkefeld. About eight days after inoculation, both animals showed unmistakable symptoms of hog cholera. Hog 630, however, exhibited a milder case than Hog 629. Eleven days after inoculation Hog 630 quickly regained normal condition, rise in temperature subsided by crisis, and on July 17 the animal was pronounced normal. Subsequent exposure with control Hog 641 (see p. 482) demonstrated a condition of active immunity. Hog 629 developed a subacute case of hog cholera. The course of this disease lasted for weeks. This animal manifested typical symptoms of the disease and after July 12 the more important symptoms noted were anorexia, malaise,

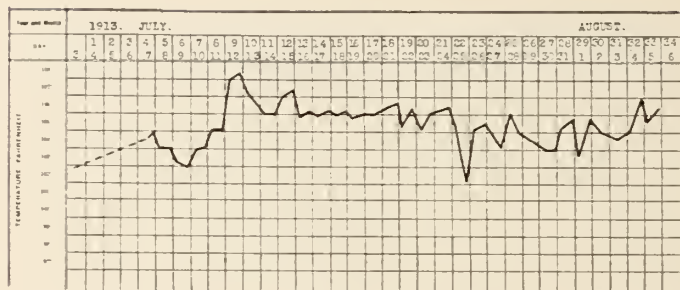
Hog 634.



- July 15. Intramuscular injection of 5 c.c. blood serum of Hog 625, diluted 1:4, and Berkefeld filtered.
 July 20–21. Inactive, poor appetite, somewhat constipated.
 July 24. Apparently normal.
 July 27–August 5. Symptoms of subacute type of cholera.
 August 6. Nearly moribund.
 August 12. Animal found dead.

Typical lesions of hog cholera in subcutaneous tissue; heart, lungs, kidney, spleen, cecum, and lymph glands.

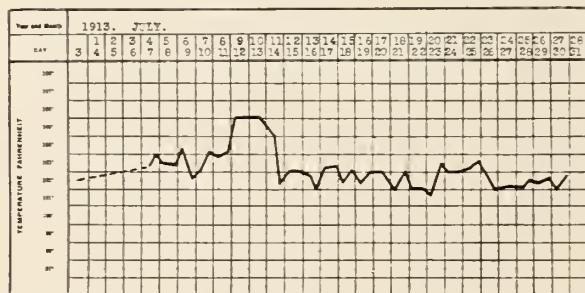
HOG 629.



- July 3. Intramuscular injection 10 c.c. filtered Culture 587 (May 27, 1913)—(July 3, 1913), 1:10. Berkefeld filtered.
- July 9. Appetite poor.
- July 10-20. Symptoms of subacute type of cholera: anorexia, blepharitis, malaise, roughened coat, emaciation, and profuse diarrhea.
- July 24. Improvement noted.
- July 28. Relapse.
- August 6. Nearly moribund.
- August 7. Animal found dead.

Lesions: heart, ecchymosis on auricles; lungs, complete consolidation; spleen, enlarged, soft, friable; hemorrhagic; kidney, few petechiae; cecum, ulcers near ileocecal valve; inguinal and other lymph glands, enlarged, hemorrhagic; adhesions of ascending colon to peritoneum; extravasation in subcutaneous tissue. *Spirochaeta suis* present.

HOG 630.



- July 3. Intramuscular injection of 10 c.c. filtered Culture 587 (May 27, 1913)—(July 3, 1913), 1:10. Berkefeld filtered.
- July 8. Poor appetite.
- July 9-14. General inactivity, poor appetite, no apparent emaciation or loss of general tone.
- July 15. Appetite good.
- July 17. Apparently normal.
- July 31. Animal normal. Released.

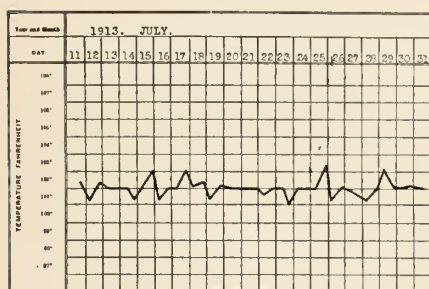
blepharitis, emaciation, and roughened coat. Hog 629 was found dead on August 7, 35 days after inoculation.

As a control on this experiment, Hogs 629 and 630, on July 11, were transferred from the room which they had occupied in the experimental stable to another, and, after disinfection of the room, normal Hogs 632 and 633 were immediately placed in the quarters formerly occupied by sick Hogs 629 and 630. An examination of the dates on the clinical charts will show that this rearrangement was made during the height of the disease in Hogs 629 and 630. Hogs 632 and 633 were subjected to exactly the same conditions as those attending the care and treatment of Hogs 629 and 630 except, of course, that they received no inoculation with infectious material. These control animals remained perfectly normal in every way and after being kept as controls for some time were utilized in experiments.

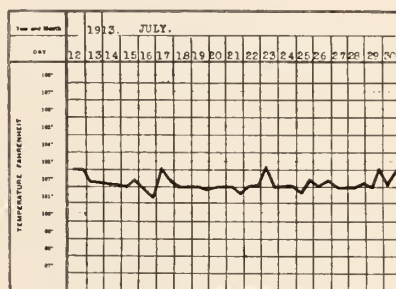
Hogs 636 and 637 on July 15 each received an intramuscular injection of 5 c.c. of the defibrinated, diluted, and Berkefeld-filtered blood serum of Hogs 629 and 630, respectively. Both of these animals developed typical symptoms of hog cholera

Hog 632.

Hog 633.



Normal hog.



Normal hog.

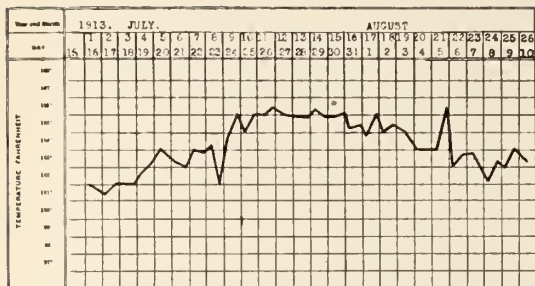
and both were moribund 26 days after inoculation. Practically the only difference observed in their clinical appearance was that Hog 636, which had received the filtered blood from Hog 629, showed more severe symptoms of the disease after a shorter incubation period than those manifested in Hog 637, which had been inoculated with the blood from Hog 630. This result was consistent, as the injection of the filtered culture described above into Hog 629 produced the subacute type of hog cholera, while Hog 630, after a marked reaction, quickly regained normal condition.

In order to make the present experiment more complete Hogs 639 and 640 were injected each intramuscularly with 4 c.c. of suspension of Culture Transfer 2-587-B, which, after 53 days' incubation, had been macerated in sterile water, diluted 1:6, and subjected to filtration through the Berkefeld.

From the fifth to the ninth day following inoculation, Hog 639 showed a marked reaction. During this period the animal was somewhat inactive and had poor appetite. Nine days after inoculation Hog 639 regained normal condition.

Hog 640 manifested no apparent reaction from the inoculation. This was regarded as an indication of natural immunity, as Hog 640 is a large brood sow and is probably a "natural immune."

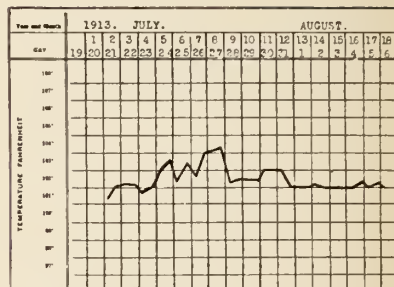
Hog 636.



- July 15. Intramuscular injection of 5 c.c. blood serum of Hog 629, diluted 1:4, and Berkefeld filtered.
 July 21. Animal "off feed" and constipated.
 July 22-August 5. Typical symptoms of subacute type of cholera.
 August 6. Animal nearly moribund.
 August 11. Animal killed.

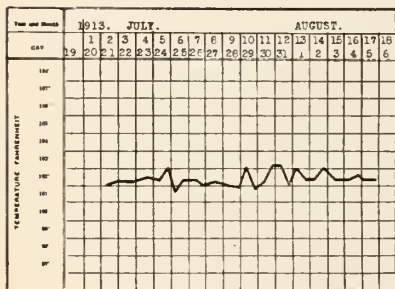
Typical lesions of hog cholera in lungs, spleen, kidney, cecum, and lymph glands.

Hog 639.



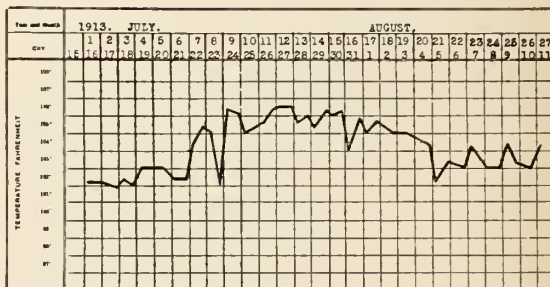
- July 19. Intramuscular injection of 4 c.c. suspension Culture 587 (May 27, 1913), dilute 1:6, and Berkefeld filtered.
 July 24-28. Anorexia, inactivity.
 July 29. Normal.

Hog 640.



- July 19. Intramuscular injection of 4 c.c. suspension, Culture 587 (May 27, 1913), diluted 1:6, and Berkefeld filtered.

Hog 637.



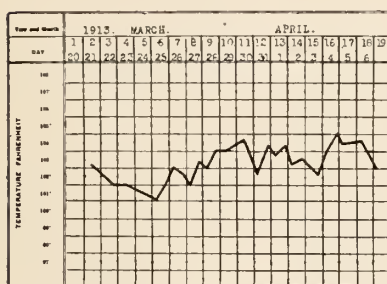
- July 15. Intramuscular injection of 5 c.c. blood serum of Hog 630, diluted 1:4, and Berkefeld filtered.
 July 21. Appetite poor; constipated.
 July 22. Anorexia, constipation and diarrhea, weakened condition, emaciation.
 August 6. Animal nearly moribund.
 August 12. Animal killed.

Typical lesions of hog cholera; lungs, kidney, lymph glands, mucosa of cecum congested.

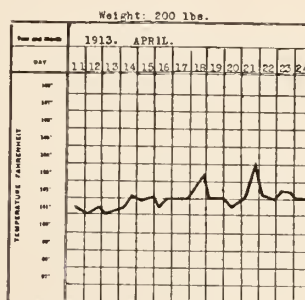
V. UNSUCCESSFUL INOCULATIONS WITH CULTURES, CONTROL ON POSITIVE RESULTS.

a) *Strain 576*.—Hog 576, on March 20, was inoculated intramuscularly with 5 c.c. of hog cholera virus, strain Kansas III. This animal developed typical symptoms of cholera and, being moribund 18 days after inoculation, was bled and examined. The ordinary lesions of hog cholera were present and the material from the ulcers in the cecum, when examined by the dark field method, showed numerous spirochetes and spirilla of various forms. From this material in the ulcers, cultures were made, and after three days' incubation some of these cultures were submitted to dark field examination. On account of the fact that some large forms of spirochetes were present, which at that time were regarded as possibly the same form which had repeatedly been found in the blood of cholera hogs, Culture 576-A was macerated in physiologic salt solution and 5 c.c. inoculated intravenously into Hog 584.

Hog 576.



Hog 584.



March 20. Intramuscular injection of 5 c.c. Kansas III virus.

March 29. Animal not eating, general symptoms appearing.

April 4. Animal weak.

April 8. Moribund, bled from the carotid artery.

Typical lesions: lungs, heart, spleen, lymph glands; ulcers in cecum.

April 11. Intravenous injection of 5 c.c. physiologic salt suspension, Culture 576.

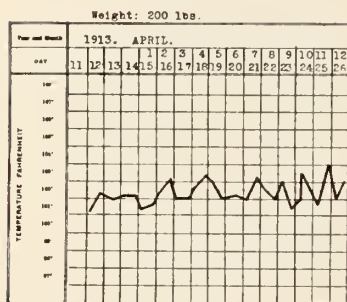
Hog 584, which received Culture 576-A, showed a slight thermal reaction on April 18, seven days after injection with the culture. Again on April 21, the temperature rose two degrees. These thermal reactions were possibly due to the presence of a variety of contaminating organisms in the suspension, as no clinical symptoms were apparent in the animal. On April 25 the animal was released as normal and subsequent inoculation with hog cholera serum showed that Hog 584 was susceptible to the disease.

From the original culture, 576-A, transfers were made on April 11 and incubated under anaerobic conditions. Transfer 1-576-A, six days after inoculation, on being submitted to dark field examination, showed the presence of relatively large spirochetes, similar to those which had been found in the original culture, 576-A. Therefore, Transfer 1-576-A was suspended in physiologic salt solution and 2.5 c.c. of the suspension injected intravenously into Hog 585, on April 14. Five days after inoculation

the animal showed an irregular temperature ranging between 102° and 103° , and this variation in thermal condition was present for several days following. However, no symptoms of hog cholera were apparent and it was concluded that the fluctuating temperature was possibly due to the injection of contaminating organisms which were present in Transfer 1-576-A. The animal was finally released, and on being injected later with horse serum virus, hog cholera was produced and death eventually resulted.

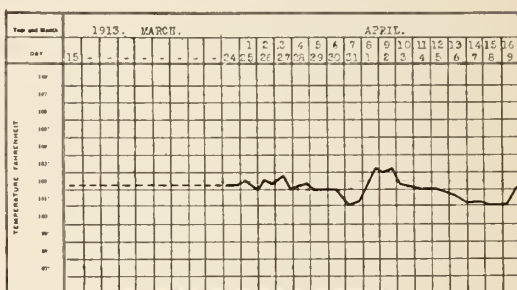
b) *Strain 560*.—On February 14 Hog 560 received 5 c.c. of Kansas III virus intramuscularly. Six days after inoculation the animal developed symptoms of acute cholera and after a duration of the disease for five days, being moribund, was bled and examined. Typical lesions of cholera were found on autopsy. Numerous spirochetes of various forms were found in the necrotic material contained in the ulcers of the cecum. Inoculations into culture media were made in the usual way and after five days' incubation one of these cultures, designated as "Culture 560-A," after being

Hog 585.



April 14. Intravenous injection of 2.5 c.c. physiological salt suspension, Transfer 1, Culture 576.

Hog 571.



March 15. Blood normal.

March 15-24. Animal normal.

March 24. Intravenous injection of 2 c.c. physiological salt suspension, Culture 1, Transfer 2, Formol (March 3, 1913), Hog 560.

March 31. Animal normal.

April 3. Animal a little "off feed."

examined on the dark field, was found to contain a growth of spirochetes. Contamination with various forms of bacteria and cocci, however, was present. In an attempt to eliminate as much of this contamination as possible, Culture 560-A was transferred into various culture media containing different chemicals, for the purpose of exerting slight germicidal action and the possible inhibition of the extraneous organisms. On March 24, after 21 days' incubation, Transfer 1-560-A, which had been made in a horse serum medium containing a small percentage of formol, was found, on dark field examination, to contain a few spirochetes, but no granules were observed. Two cubic centimeters of a physiologic salt suspension of Transfer 1-560-A were inoculated intravenously, March 24, into Hog 571. This animal showed no apparent result following the inoculation of this culture, except for a slight reaction which occurred during the eighth and ninth days after the inoculation. Subsequent injection of Hog 571 with horse serum virus resulted in the death of the animal from hog cholera of the acute type.

This includes all of those experiments which proved unsuccessful, and serve as admirable controls on the results given. It will be noticed that the unsuccessful experiments included under the last heading were conducted during the months of March and April. At that time it had not been definitely determined that the crypts of the ceca of normal hogs may contain spirochetes and spirilla. In so far as our observations go, these organisms are usually relatively large and altogether unlikely to be related to the form which we have designated as *Spirochaeta suis*, the latter being found in the intestinal ulcers and the external local lesions of cholera hogs. No doubt, the small spirochete described in the first part of this paper as *Spirochaeta suis* was present in the ulcers of Hogs 576 and 560, this material being used in the inoculation of the cultures described under the present heading. However, all of the inoculations into hogs, of cultures from these two strains, were made after a comparatively short period of incubation. This explains the failure to produce positive symptoms of hog cholera with the cultures, as our later results reported above indicate that it is necessary to incubate the cultures for several weeks in order to grow *Spirochaetae suis*.

The most important bearing, however, of these unsuccessful attempts to produce the disease with the cultures noted under the last heading pertains to the control which they give on the results described in the preceding pages. The foregoing positive results from inoculations of filtered and unfiltered cultures of *Spirochaeta suis* are lacking in one detail. Without some means of controlling the results, it might be logically assumed that those animals which had received hog cholera from the injection of cultures and transfers did so as the result of the injection of a small amount of original virus, which possibly might have been transferred through the cultures. This we know to be untrue because of the failure to produce hog cholera in animals inoculated with cultures and transfers from Strains 576 and 560. Positive results followed the inoculation of Hog 559 with the original culture, 551-A, which had an incubation period of only five days. The result may have been due to some of the original virus having been transferred to the animal through the culture. However, we believe that the



Hog 559.—Inoculated 22 days previously with suspension of culture from cecum of Hog 551.

succeeding results are sufficiently controlled by the facts that Hog 584 failed to develop hog cholera after inoculation with Culture 576-A, and that Hogs 571 and 585 did not respond to the injection of Transfers 1-576-A and 1-560-A, respectively.

CONCLUSIONS.

Spirochaeta suis is an organism found in the intestinal ulcers, crypts in the ceca, and external local lesions of animals suffering from hog cholera. It is a typical spirochete, simulating in many characteristics *Spirochaeta pallida*, *Spirochaeta gallinarum*, and other forms whose morphology and life history are becoming better understood. *Spirochaeta suis* appears to be capable of breaking up into granules and these granules may play an important part in the life cycle and physiological functions of the organism. They are present in the blood of cholera hogs, in cultures of the *Spirochaeta suis*, and are capable of producing the disease in healthy hogs.

In the blood of hogs suffering from cholera the presence of a relatively large spirochete in few numbers has been recognized. As this organism has not been found in the blood of normal hogs it may represent *Spirochaeta suis* in a transitional form. Its morphological variation from *Spirochaeta suis*, as found in ulcers and local foci, may be due to the unfavorable action of blood as a medium or to its natural processes as a part of the life cycle of the species.

Spirochaeta suis is an obligatory anaerobic organism and usually requires several weeks' incubation for growth to take place on artificial culture medium. It may be transferred from generation to generation on artificial culture medium. Cultures containing the organism in the form of granules and spirochetes may be passed through bacteria-proof filters and the spirochetes removed, the few small granules which pass through being capable of producing hog cholera or resistance to the disease.

Spirochaeta suis is capable of producing typical hog cholera when injected into healthy hogs. This is true not alone of contaminated cultures made directly from the intestinal ulcers of cholera hogs; second and third generations on artificial culture media, containing the *Spirochaeta suis*, as well as the Berkefeld

filtrates of the same transfers, are capable of producing hog cholera and marked reactions, which confer more or less protection against the disease. The pathogenicity of these cultures does not appear to be due to the passage of an unknown "invisible microorganism" which is finally transmitted to healthy hogs by inoculation. Control experiments tend to show that the pathogenicity of the cultures of *Spirochaeta suis* is due to the species itself in the form of spirochetes or granules.

Finally, in those hogs which receive the disease from cultures of *Spirochaeta suis*, the organism is present in the intestinal lesions or local external lesions, as demonstrated by the dark field examination.

From the above results, which have practically fulfilled Koch's laws, in so far as it is possible with an organism possessing the biological characteristics of spirochetes, it may logically be concluded that *Spirochaeta suis* is more nearly established as the specific cause of hog cholera than any other known organism.

THE ANTIGENIC PROPERTIES OF THE CONSTITUENTS OF THE PNEUMONIC EXUDATE.*

SERUM STUDIES IN PNEUMONIA. II.

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In a previous communication,¹ some observations were reported concerning the antigenic properties of normal fibrin and fibrin from patients having pneumonia to normal serum and serum from pneumonia patients. In brief, the main conclusion drawn at this time was that human serum (both normal and pneumonic) sometimes contains antibodies for human fibrin, which, in part, may be responsible for the dissolution of the fibrinous exudate in pneumonia.

The present report is a continuation of the studies suggested by the earlier experiments. Altho there are some points here discussed which lead us from the main trend of the work and which do not have a direct bearing upon conditions in pneumonia, they will be included, since they are interwoven with the methods used in the experiments and may aid in explaining some of the reactions observed. The same methods of securing fibrin and of carrying out the hemolytic tests were used here as in the previous work.

Since only a few specimens of fibrin showed antigenic properties to human serum, several methods to enhance this antigenic power were tried. One was to add varying amounts of a fat or lipid solution to the fibrin. The fats used were recovered from pneumonic lungs by Dr. Oskar Klotz. The strength of the solution was the same as that used in the Noguchi test (0.3 gm. in 10 c.c. alcohol and ether [9-1], and this diluted 1 in 10 with salt solution). This dilute solution was itself found to be hemolytic in amounts of five drops. It was only slightly anticomplementary and in the presence of some sera proved to be antigenic. This antigenic property of the fats was not constant for pneumonic, normal, or positive syphilitic serum.

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¹ *Jour. Infect. Dis.*, 1913, 13, p. 69.

No difference in the antigenic power of fibrin with and without the addition of the fats was found in the presence of 25 different sera, 17 being from pneumonia and 8 from normal individuals. The anticomplementary power was greatly increased by the presence of both fibrin and fats, as will be shown later.

Thirty-six specimens of fibrin (18 pneumonic and 18 controls) were used in testing the hemolytic property of the powdered fibrin suspended in salt solution. Among the 18 specimens of fibrin from pneumonia patients, 4 were found to be hemolytic. None of the controls showed this power. This power on the part of powdered fibrin to produce hemolysis was very decided when present.

The fatty substances isolated from pneumonic lungs by Dr. Oskar Klotz have been found to be very hemolytic, five drops of the 1-10 dilution in salt solution causing complete hemolysis of 1 c.c. of a 1 per cent suspension of human red cells in three hours. The fibrin suspension used as antigen was found to inhibit this hemolysis by fats when used in fairly large quantities (8-10 drops). There was evidence of a slight variation in the power of the fibrin to inhibit the hemolysis, but this was thought to be within the limits of variance of the strength of the suspension. Several specimens of fibrin from cases of pneumonia which in themselves were hemolytic did not inhibit this hemolysis and even after the extraction of the hemolytic principles by alcohol they did not inhibit the hemolysis in 25 drops (two and one-half times the amount of normal fibrin necessary completely to inhibit hemolysis). Proof of the extraction of the hemolytic principles from the fibrin lay in the finding of a hemolytic power in the alcoholic extract while the residue remained inactive.

In a series of experiments it was noted that human sera, whether normal or pneumonic, in small quantities inhibited the hemolysis by these fats. Consequently some further observations were made whereby it was shown that simple dilution with salt solution did not inhibit the hemolysis by the fats, while serum in sufficient quantities to prevent hemolysis did so regardless of the dilution (1-50). To determine whether heat had any appreciable effect upon this antihemolytic quality of the serum, several portions of

a serum were treated at various temperatures—37° C. for 20 hours; 56° C. for 20 hours; 65° C. for 30 minutes; 75° C. for 30 minutes; 90° C. for 30 minutes—and afterward tested. No diminution in the antihemolytic power of the serum was found.

The protein fraction of the serum possessing this inhibitive power was then determined. The method employed by Quinan¹ was used for the fractionation of the serum. Ten cubic centimeters of normal serum (inactivated at 56° C. for 30 minutes) were diluted with 100 c.c. of distilled water. Carbon dioxid was passed through the diluted serum to saturation, causing a white, flocculent precipitate (globulins insoluble in water). This precipitate was washed thoroughly with distilled water and dissolved in 10 c.c. of saline (Solution 1). The washings and filtrate were combined and reduced by evaporation to approximately the original volume. Magnesium sulfate was added to saturation and the whole allowed to stand over night. A white precipitate appeared which was filtered off (globulin soluble in water). This precipitate was washed with a saturated solution of magnesium sulfate and redissolved in a small amount of water. Barium hydroxid was added in excess, causing a heavy white precipitate (barium sulfate and magnesium hydroxid). The precipitate was removed by filtration. The filtrate was saturated with carbon dioxid and absolute alcohol gradually added. A white flocculent precipitate separated out (water-soluble globulin). The precipitate was washed with absolute alcohol, dried with ether, then redissolved in saline (Solution 2).

The filtrate from the magnesium sulfate precipitation was dialyzed in running water for 18 hours. The remaining solution contained the albumins, (Solution 3).

Each fraction was added in increasing quantities to constant hemolytic quantities of fat solution and red blood cells. The antihemolytic power of the water-insoluble globulin (Solution 1) was found to be practically equal to that of the whole serum. There was very little or no inhibitive power present in the other two fractions, the water-soluble globulin and albumin.

To determine whether the hemolytic principles were confined to any one fraction of the fats, they were fractionated according

¹ *Univ. of Calif. Pub. (Pathology)*, 1903, 1, p. 1.

to the method used by Noguchi and Bronfenbrenner.¹ The results here given of the four fractions include also the tests of their anticomplementary power.

Fraction	Hemolytic	Anticomplementary
1. Ether insoluble—hot alcohol insoluble.....	—	—
2. " " — " " soluble.....	++	—
3. Ether soluble—acetone insoluble.....	+	—
4. " " — " " soluble.....	+	—

Since leukocytes form such a large portion of the pneumonia exudate it was thought that they, or their decomposition products, might lead to the production of antibodies in the blood. The leukocytes were obtained from the centrifuged specimens of defibrinated blood from two cases of pneumonia, and from several abscesses and empyemas opened at operation. The organisms present in the pus were the pneumococcus, staphylococcus, or streptococcus. The leukocytes, as well as the pus, were washed thoroughly with salt solution. The leukocytes from the cases of pneumonia were immediately extracted by distilled water at 37° C. The specimens of pus were divided into portions, one being placed in 50 per cent pure glycerol, the other repeatedly extracted with alcohol and dried with ether. When used, the leukocytes were extracted by distilled water over night at 37° C., those from glycerol being thoroughly washed with saline. Chloroform, xylol, or toluol was added to the distilled water and leukocytes to prevent bacterial decomposition. The sediment was thrown down by the centrifuge.

The watery leukocytic extracts obtained in this way varied considerably; some were clear, others quite opalescent, some were hemolytic, others were not. They were not anticomplementary in small amounts and did not serve as complement. When used as antigen no constant results were obtained in regard to pneumonia sera, as is shown by the following results: The leukocytic extracts in the presence of the serum from 5 cases of pneumonia showed binding of complement; in the presence of 14 others there was no binding. In the presence of 9 of the control sera (3 with positive reactions to the Noguchi test) there was binding of complement, and in the presence of 18 other controls (5 with positive reactions to the Noguchi test) there was no binding.

¹ *Jour. Exper. Med.*, 1911, 13, p. 43.

An interesting observation was made in the course of these experiments, in that binding occurred in the presence of all of six sera used in the tests with the extract of the cells from the two cases of pneumonia. Four of the sera were from pneumonia patients and two from controls.

One specimen of leukocytes (from pus) kept in 50 per cent glycerol when washed and resuspended in saline was not anti-complementary in small amounts, nor did it prove antigenic in the presence of one pneumonia and one normal serum. The leukocytic extracts when added to fibrin as antigen did not increase the antigenic power of the fibrin.

The leukocytic residue after extraction by water was washed in saline and used as antigen. This residue did not prove anti-complementary in small amounts. When it was used as antigen, the results were varying. The residue in the presence of one pneumonia serum bound complement; in the presence of eight other pneumonia sera there was no binding of complement; in the presence of six control sera there was binding, and in the presence of eleven other control sera there was no binding of complement. Of the control sera showing binding, one showed a positive and four a negative reaction to the Noguchi test; among those showing no binding, six showed a positive and four a negative reaction to the Noguchi test.

It was found that the residue of the cells which had been kept in 50 per cent glycerol previous to extraction was hemolytic, while that from previously dried leukocytes was not. By subjecting the hemolytic cell masses to the action of alcohol, it was found that the hemolytic principles disappeared and were demonstrated in the alcoholic extract. The residue, after the alcoholic extraction, was anticomplementary when used in large amounts, a fact which is also true for other leukocytic débris.

The nature of the fats from leukocytes was determined in a manner similar to that for the fats from pneumonic lungs. Several hundred cubic centimeters of thick, creamy pus from an abscess were incubated under toluol for several days at 37° C. Considerable change due to digestion occurred, making the pus very much more fluid. The whole was filtered and the remaining thick,

greasy residue was extracted repeatedly with alcohol, then ether. The alcoholic and ethereal extracts were mixed and filtered until a clear solution was obtained. This was evaporated to dryness, leaving a thick, dark brown, fatty residue, which was divided into several portions for study.

One portion was used to test its hemolytic, anticomplementary, and antigenic power. It was found to be very hemolytic and not anticomplementary and served inconstantly as antigen in the presence of some sera.

In a series of experiments corresponding with those carried out with the other fats it was found that normal fibrin in fairly large quantities (5-10 drops) inhibited the hemolysis by the leukocytic fats. Serum was also found to inhibit this hemolysis.

An interesting observation was made when using the fat solution, the fat solution and fibrin, and the fat solution and serum with the hemolytic system. There was no anticomplementary action by the fat solution alone, the fibrin alone, or the serum alone, while increasing amounts of fat solution in the presence of a constant amount (2 drops) of normal fibrin suspension and the hemolytic system showed three reactions in regard to the hemolysis: (1) in which the fibrin and fats were in too small a quantity to inhibit the hemolysis by the hemolytic system; (2) in which the amounts of fibrin and fat solution were sufficient to inhibit completely the hemolysis by the hemolytic system; and (3) in which the fats were in sufficient excess to cause hemolysis by their own action, tho enough fibrin and fat solution were present to inhibit the hemolysis by the hemolytic system. These same reactions occurred in the presence of both normal and pneumonic serum.

Another portion of the leukocyte fats was fractionated in the same manner as the fats from pneumonic lungs and somewhat similar results found in regard to their hemolytic and anti-complementary power.

Fraction	Hemolytic	Anticomplementary
1. Ether insoluble—hot alcohol insoluble.....	—	—
2. " " " " soluble.....	+	—
3. Ether soluble—acetone insoluble.....	++	+
4. " " " " soluble.....	+++	—

These four fractions were tried with a large number of sera in testing their antigenic properties. It was found that an occasional serum was positive with several portions and without regard to the result of the Noguchi test, so that any fraction may prove antigenic in the presence of some sera.

DISCUSSION.

Since the suggestion appeared in the first paper associating the antigenic property of the fibrin with fatty substances, it has been shown in our experiments that by artificial means this antigenic property of the fibrin cannot be increased by subjecting the fibrin to solutions of fatty substances. The addition of certain quantities of lipid substances to the fibrin suspensions increased the anti-complementary property of each, a point which will be discussed again.

That a few specimens of fibrin from pneumonia patients should prove to be hemolytic is rather curious. This power is evidently due to associated fatty substances, since this property can be removed by extracting the fibrin with alcohol and it is then present in the alcoholic extract. It is perhaps true that all the specimens of fibrin used were accompanied by a greater or less quantity of fats derived, perhaps from the leukocytes, during the process of clotting. The quantity of fat present was much greater in some than the average. Why this quantity of fatty substance bound to the fibrin should be greatly increased in some cases of pneumonia is not clear, tho, if it is derived from the leukocytes, it may be the result of the increased quantity of fatty substances known to occur in the leukocytes during pneumonia and may be merely an adhesion of the fats to the fibrin.

Fatty substances extracted from the body tissues are known to be hemolytic and Lamar¹ has shown the inhibitory effect of serum upon this hemolytic action. That this inhibitive power should be limited to the one fraction of globulins is interesting and is another indication of the activity and importance of the globulin content of blood serum. Heating the serum does not interfere with this power to inhibit the hemolysis by the fats.

¹ *Jour. Exper. Med.*, 1911, 13, p. 1.

Fibrin which in itself does not cause hemolysis will inhibit the hemolysis by fatty substances. It is interesting that a specimen of fibrin from a patient with pneumonia, which had proved to be hemolytic, when extracted by alcohol lost its power to inhibit the hemolysis by the fats. The removal of the associated fats by the alcohol would not effect this change so that a change in the fibrin itself must have been produced unless the alcohol removed substances other than the fats.

The fats isolated by the digestion of a quantity of pus were mainly of leukocytic origin, as the bulk of the pus was mainly made up of leukocytes, there being little serum present. These fats closely resembled those isolated from the pneumonic lungs. They were hemolytic and, in the presence of some sera, antigenic. This antigenic power bore no relation to the reaction to the Noguchi test. When fractionated into four fractions similar to those of the fats isolated from the pneumonic lungs, they proved to have similar properties. When these fractions were used as antigen it was found that any fraction might prove antigenic in the presence of some sera regardless of the reaction of these sera to the Noguchi test. This finding adds to the large number of substances capable of binding complement in the presence of serum.

Hiss and Zinser¹ have pointed out the beneficial effect of leukocytic extracts in the treatment of a number of infections including pneumonia. To what action this beneficial effect is due is not known. More pronounced results were obtained by their administration than by that of leukocytes or immune serum. The occurrence of a reaction to the treatment by extracts suggests the development of an immunity to them. Manwaring² showed the presence of bactericidal substances in leukocytic extracts which apparently are different from those in the serum, a point maintained by Schattenfroth³ and Daeubler.⁴ Buchner and Hahn⁵ believe the bactericidal substances to be identical with alexine. Petterson⁶ showed that the leukocytes and bone marrow are much more bactericidal in an immunized animal than in normal ones and claims that the bactericidal substances are not secreted by the

¹ *Jour. Med. Research*, 1908, 19, p. 321.

² *Jour. Exper. Med.*, 1912, 16, p. 249.

³ Quoted by Petterson.

⁴ *Ibid.*

⁵ *Ibid.*

⁶ *Centralbl. f. Bakteriol.*, I, Orig., 1905, 42, p. 56.

leukocytes but retained in them until injured. From our results it does not appear that leukocytes act as antigen, as we were unable to demonstrate a complementary antibody with any greater frequency in pneumonic sera than in controls.

It is interesting that the extract of the leukocytes from the two cases of pneumonia bound complement in the presence of all the sera (4 pneumonic and 2 control) with which it was used. It may indicate that some constituents other than fats in the leukocytes in pneumonia have a greater affinity than normal for complement or that these elements are increased in this disease. The great outpouring of leukocytes into the lungs would offer a means of attracting more complement into the alveoli where it would aid in the destruction of the infecting organisms and in the removal of the exudate.

The leukocytic extracts varied, no doubt, greatly in their content, but, as Hiss and Zinser have experienced, there is no method of standardizing them nor of controlling their constancy. They have never served as complement and may prove quite hemolytic. They are anticomplementary in large amounts, but the difference between the amount used in the hemolytic tests and that used to cause binding of complement is so great that the binding in the presence of the serum cannot be due to this anticomplementary action alone. In this extraction the fats are left behind so that the constituents of the extracts are, no doubt, principally proteins.

The question of the reactions reported in these papers being due to the summation or accentuation of the anticomplementary power of the reagents has been carefully considered. It is true that the fibrin, leukocytic extracts and residues, and the fatty substances used as antigen in the experiments are in large quantities capable of preventing the co-operative action of complement with amboceptor. In the quantities used there was not sufficient present to cause any appreciable effect upon the hemolysis by complement-amboceptor action. The sera used showed no evidence of anticomplementary power in quantities at least twice as large as those used in the tests. It is true according to Noguchi²

² *Loc. cit.*

that the addition of one substance to another, both of which are very slightly anticomplementary, causes an increase in the anticomplementary action of the combination of the two. This increased anticomplementary power is very well shown where the addition of the fat solution to fibrin and to serum in proper proportion causes complete inhibition of the hemolysis by complement amboceptor action, neither of the substances proving anticomplementary in amounts much greater than those used in the experiment. If this same phenomenon occurs between the serum and the antigen used in other fixation tests, an endeavor must be made to measure the anticomplementary power of the combination of the two in order to correctly read the true binding of complement by the specific reaction between serum and antigen. In spite of this evidence of marked anticomplementary power of some combinations of antigens and sera, we feel that the reactions under discussion as binding of complement are true instances of that reaction.

A most obvious omission in these reports is the lack of consideration of the causative factor—the pneumococcus. As a word of explanation we would say that, since there is much hesitancy in accepting the pneumococcus as the center around which all the phenomena of crisis and resolution occur, it appeared desirable for us to study some of the phenomena associated with equally prominent constituents of the lung exudate. We have used each of the important constituents of the pneumonic exudate as antigen, alone and in combination with other substances, in an effort to discover a relationship to processes of immunity developed against them. Most of the results have proved negative. The frequency of non-specific binding of complement by the interaction of various antigens and sera is striking and emphasizes the necessity for very careful interpretation of all such reactions and the necessity for thorough control of all factors.

CONCLUSIONS.

1. The addition of fatty substances and leukocytic extracts to fibrin does not increase its antigenic power.
2. Leukocytic extracts, leukocyte residues, fatty substances from pneumonic exudates and from leukocytes (pus cells) cause

non-specific binding of complement in the presence of some sera regardless of the reaction to the Noguchi test.

3. Some specimens of pneumonic fibrin are themselves hemolytic.

4. Fatty substances from pneumonic lungs and from leukocytes are hemolytic, but not anticomplementary in small quantities.

5. Normal fibrin and the non-hemolytic pneumonic fibrin, human serum, normal as well as pneumonic, inhibit the hemolysis induced by these fatty substances.

6. The inhibitive power of the serum is contained in the globulin fraction precipitated by carbon dioxid.

7. The four fractions of the fatty substances from pneumonic lungs and from leukocytes simulate those used as syphilitic antigen studied by Noguchi and Bronfenbrenner in their hemolytic, anticomplementary, and antigenic powers.

8. The leukocytic extracts vary to a considerable degree in their hemolytic, anticomplementary, and antigenic properties. They do not serve as complement.

9. The residues of leukocytes kept in glycerol are hemolytic and the hemolytic principles may be extracted by and demonstrated in alcohol.

10. Extracts of leukocytes from patients with pneumonia are more strongly antigenic than those from pus cells.

11. The hemolytic substances associated with some pneumonia fibrin can be removed by alcohol, the alcoholic extract proving hemolytic.

12. The anticomplementary power of normal fibrin is greatly increased by the addition of the fatty substances used in the experiments.

My thanks are due to Dr. Oskar Klotz for his constant assistance, and to Dr. McMeans for furnishing materials.

A STUDY OF THE LESIONS PRODUCED BY BACILLUS PROTEUS.*

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The proteus group of microorganisms has interested bacteriologists since the publication of Hauser's work on this family. Altho Hauser and others have described various strains belonging to this group of microorganisms, there seems to be little or no unanimity of opinion with reference to the biological characters by which the various strains may be recognized and differentiated.

The question as to the pathogenic rôle which the proteus bacilli play in human and veterinary pathology is still unsettled. Metchnikoff believes *B. proteus* to be the cause of infantile diarrhea. C. O. Jensen found it to be the cause of a form of diarrhea of calves. Meat poisoning, Weil's disease, peritonitis, and pleurisy have all been ascribed to this group of microorganisms.

We became interested in *B. proteus* in March, 1912, when the organism was repeatedly isolated in pure culture from the wound of a patient at the University Hospital, who had undergone nephrectomy because of tuberculosis of the kidney. The organism proved to be pathogenic for rabbits and rats even when injected in very small doses. Since then we have isolated *B. proteus* in pure culture in two other cases of human infection in which it was found to be highly pathogenic for rabbits.

The experimental lesions produced by *B. proteus* vary widely in character since they depend upon the virulence of the particular culture employed. Very virulent cultures introduced into the peritoneal cavity of rabbits, rats, or guinea-pigs produce death within a few hours, evidently because of the intense toxemia. In these cases there is a little increase of fluid in the peritoneal cavity and some congestion of the peritoneal surfaces.

When less virulent cultures are inoculated intraperitoneally or subcutaneously, the animal undergoes rapid and extreme emacia-

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tion. Death usually occurs within one week from the intraperitoneal inoculation, and somewhat later from subcutaneous inoculation. The peritoneal surfaces show a number of whitish nodules of varying size. Histologically, the nodules in these acute cases consist almost entirely of polymorphonuclear leukocytes associated with large numbers of bacteria. Frequently the center of the nodule undergoes liquefactive necrosis. From the subcutaneous inoculation a single abscess develops, gradually increasing in size until death occurs.

When cultures of low virulence are injected intraperitoneally, numerous firm, whitish nodules develop on the peritoneal surfaces within a few days. The animals often undergo a progressive loss of weight which terminates in death or in ultimate recovery. In the milder infections there may be no loss of weight. Histologically, the nodules show a chronic type of inflammation largely proliferative in character. It is a granuloma. A large number of connective tissue cells rich in cytoplasm (epithelioid cells) appear. Many multinucleated cells are often seen, but no giant cells of the Langhans type have been found in the experimental lesions. A varying number of large and small mononuclear leukocytes occurs. Some polymorphonuclear leukocytes may be present. There is often a central necrotic mass surrounded by the epithelioid cells and leukocytes. Sometimes the resemblance to a typical tubercle is close.

One of the clinical cases referred to above was a rather severe infection of a finger. Pieces of excised tissue showed typical tubercles. A proteus virulent for rabbits was recovered in pure culture from the lesion. Altho no acidfast bacilli were demonstrated, it is probable that tuberculosis was a complicating infection in this case.

In a general way it may be said that virulent cultures of *B. proteus* produce typical abscesses, and that cultures of moderate virulence produce proliferative lesions which may be classed with the granulomata. Extreme emaciation is one of the characteristic results of severe proteus infection in rabbits.

A detailed account of our experiments will be published later

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